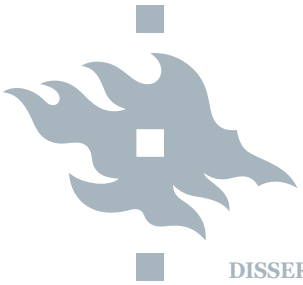

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Synthesis of Abietane-Type Diterpenoids with Anticancer Activity

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Synthesis of Abietane-Type Diterpenoids with Anticancer Activity

Laura Kolsi

ACADEMIC DISSERTATION

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“You must always have faith in people.
And most importantly, you must always have faith in yourself”
-Elle Woods

Abstract

Natural products and their derivatives are excellent starting points in drug design and widely studied as promiscuous anticancer agents. Moreover, they represent a big part of the current anticancer drugs in clinical use. Pancreatic cancer is one of the most fatal cancers with an extremely low five-year survival rate and increasing incidence. This cancer is particularly hard to target because of the huge number of genetic mutations per cancer demonstrating an urgent need for new multi-target treatment strategies. The tumor microenvironment and inflammation greatly promote tumor development and metastization. Chronic inflammation is, indeed, generally recognized as one of the hallmarks of cancer and offers a great target for drug development.

This thesis focuses on the semisynthesis of abietane-type diterpenoids and studies about their anticancer and anti-inflammatory activities. The development of novel methods focusing on catalysis and sustainability within diterpenoid chemistry was also an objective of this thesis. Dehydroabietic acid, the starting material for these studies, exists in the rosin of coniferous trees and exhibits a wide range of biological activities including anticancer and anti-inflammatory activity. Moreover, due to its commercial availability, it provides a good and inexpensive starting material for the semisynthesis of complex naturally occurring abietanes and novel diterpenoids aiming towards improved bioactivities.

Benzylic oxidation of aromatic abietanes is a key chemical transformation for the functionalization of the abietane core. To replace the environmentally hazardous and noxius chromium(VI) reagents, widely used for this oxidation, we studied sodium chlorite in combination with aqueous *tert*-butyl hydroperoxide. This method enables the preparation of 7-oxo, 7-oxo-15-hydroperoxy and 7-oxo-15-hydroxy derivatives of various aromatic abietanes. For 12-substituted aromatic abietanes the method is regioselective providing the 7-oxo products in good yields. A short semisynthesis is possible with this method to obtain the naturally occurring picealactones A, B and C which exist in the heartwood of *Picea morrisonicola* Hayata and represent potential compounds for the treatment of endocrine cancers.

A 13-propenyl side chain exists in naturally occurring aromatic abietanes such as angustanoic acid E and aquilarabietic acid H, the former possessing antiviral and anti-inflammatory activities and the latter expressing antidepressant activity *in vitro*. Previous studies report the use of halogenated reagents and hazardous solvents such as benzene for the incorporation of this moeity onto aromatic abietanes. In our studies, bismuth(III) triflate was identified as the most efficient catalyst to perform the dehydration of tertiary alcohols to access this chemical entity, with high yields and good selectivity. Furthermore, by modifying the reaction conditions, namely solvent and temperature, dimerization and cyclization occurred. Expansion of the method to cover other non-terpenoid compounds showed that the method is applicable to compounds from different chemical classes. In addition, it enables a short semisynthesis of natural products from *Pinus massoniana* Lamb, presented for the first time in this work.

Overall, this thesis resulted in the synthesis of 41 new dehydroabietic acid derivatives providing new information for both diterpenoid chemistry and biology. Novel dehydroabietic acid derivatives were tested against pancreatic cancer and inflammation. *In*

vitro studies revealed that dehydroabietic oximes inhibited the growth of pancreatic cancer cells and nitric oxide production with IC_{50} values in the low micromolar range. In addition, they were able to induce cancer cell differentiation as well as downregulate cyclin D1 expression with upregulation of p27 levels, consistent with cell cycle arrest at the G1 phase. Furthermore, according to a kinase profiling study, one of the oximes showed potential in selectively inhibiting p90 ribosomal S6 kinase 2 (RSK2), an AGC kinase involved in cellular invasion and metastasis.

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Helsinki, December 2018

Laura Kolsi

Contents

List of original publications

This thesis is based on the following publications:

I Kolsi, L. E., Krogerus, S., Brito, V., Rüffer, T., Lang, H., Yli-Kauhaluoma, J. T., Silvestre, S. M., Moreira, V. M. Regioselective Benzylic Oxidation of Aromatic Abietanes: Application to the Semisynthesis of the Naturally Occurring Picealactones A, B and C. *ChemistrySelect,* 2017, **2**, 7008-7012.

II Kolsi, L. E., Yli-Kauhaluoma, J. T., Moreira, V. M. Catalytic, Tunable, One-Step Bismuth(III) Triflate Reaction with Alcohols: Dehydration Versus Dimerization. *ACS Omega*, 2018, **3**, 8836-8842.

III Kolsi, L. E., Leal, A. S., Yli-Kauhaluoma, J. T., Liby, K. T., Moreira, V. M. Dehydroabietic oximes halt pancreatic cancer cell growth in the G1 phase through induction of p27 and downregulation of cyclin D1. *Sci. Rep.*, 2018, **8**, 15923.

The publications are referred to in the text by their Roman numerals.

Author's contributions

I LEK carried out the synthesis work and optimization of the reaction conditions with the help of SK. VB conducted the biological testing and thermochemistry studies together with SMS. TR and HL performed the crystallographic studies. LEK wrote the manuscript together with VMM, SMS and JYK.

II LEK conducted all the experimental work and wrote the manuscript together with the coauthors.

III LEK carried out the synthesis and characterization of the compounds. LEK performed the biological testing and analysis together with ASL. LEK wrote the manuscript together with the coauthors.

Abbreviations

AA	abietic acid
Ac	acetyl
AGC	protein kinase A, G, and C families
ARE	antioxidant response element
BHT	butylated hydroxytoluene
Bn	benzyl
BPO	dibenzoyl peroxide
BTK	Bruton's tyrosine *kinase*
Bz	benzoyl
CAR	chimeric antigen receptor
CCL2	chemokine (C-C motif) ligand 2
CDDO	2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid
CDDO-Im	CDDO-imidazolide
CDDO-Me	CDDO-methyl ester
CDK	cyclin dependant kinase
COX	cyclo-oxygenase
CSF-1R	colony-stimulating factor 1
CTLA-4	anti-cytotoxic-T-lymphocyte protein-4
CVB	Coxsackie virus B
CXCR4	C-X-C chemokine receptor type 4
DAA	dehydroabietic acid
DCC	*N*,*N*'-dicyclohexylcarbodiimide
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DTBMP	2,6-di-*tert*-butyl-4-methylpyridine
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
ER	endoplasmic reticulum
Et	ethyl
FDA	Food and Drug Administration (U.S.)
5-FU	5-fluorouracil
GI	gastrointestinal
HeLa	human epithelial cervical
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
5-HT	5-hydroxytryptamine, serotonin
HUVECs	human umbilical vein endothelial cells
IKKβ	inhibitor of nuclear factor kappa-B kinase subunit beta
IL	interleukin
JAK	Janus kinase
Keap1	Kelch- like ECH- Associating protein 1

lncRNAs	long noncoding RNAs
MCP-1	monocyte chemoattractant protein 1
Me	methyl
MIC	minimum inhibitory concentration
MMP	matrix metalloproteinase
MRSA	methicillin-resistant *Staphylococcus aureus*
MST2	mammalian Ste20-like kinase 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBS	*N*-bromosuccinimide
NE	norepinephrine
NGF	nerve growth factor
NHPI	*N*-hydroxyphthalimide
NOESY	nuclear Overhauser effect spectroscopy
Nrf2	nuclear factor erythroid 2 related factor 2
NSAIDs	non-steroidal anti-inflammatory drugs
NF-κB	nuclear factor-κB
PAF	platelet-activating factor
p-Akt	phosphorylated protein kinase B
PCC	pyridinium chlorochromate
PDAC	pancreatic ductal adenocarcinoma
PD-L1	anti-programmed cell death 1 ligand 1
p-ERK	phosphorylated extracellular signal-regulated kinase
Ph	phenyl
PKBβ	protein kinase B beta
PMN	polymorphonuclear leukocytes
PPAR-γ	peroxisome proliferator-activated receptor γ
ROS	reactive oxygen species
RNS	reactive nitrogen species
RSK	p90 ribosomal S6 kinase
SAR	structure-activity-relationship
SO	synthetic oleanane triterpenoids
Src	proto-oncogene tyrosine-protein kinase
STAT	signal transducer and activator of transcription
SYK	spleen tyrosine kinase
TAMs	tumor-associated macrophages
TBAB	tetra-*n*-butylammonium bromide
t-Bu	*tert*-butyl
TBHP	*tert*-butyl hydroperoxide
Tf	trifluoromethanesulfonyl
THF	tetrahydrofuran
TME	tumor microenvironment
TNF-α	tumor necrosis factor α
TGF-β	transforming growth factor β
TPSA	topological polar surface area
Ts	*p*-toluenesulfonyl, tosyl

VEGF or VEGF-A	vascular endothelial growth factor (A)
VRE	vancomycin-resistant enterococci
V-70	2,2-azobis(4-methoxy-2,4-dimethylvaleronitrile)
XO	xanthine oxidase

1 Introduction

Natural products and their derivatives continue to act as an important source and inspiration for modern drug discovery,[1] especially for the treatment of cancer. About eighty percent of all approved small molecule anticancer drugs between 1981 and 2014 were natural products or their derivatives and mimicks.[2] At the moment, cancer is the second leading cause of death in Europe and in the US and the global burden of cancer is constantly increasing.[3] Pancreatic cancer is one of the most devastating cancers with an increasing incidence, high death rates and extremely low 5-year survival rates. It is suggested that it will become the second leading cause of cancer-related death before 2030.[4] Difficulties to target pancreatic cancer and chemoresistance are some of the reasons hampering chemotherapy as well as the development of new drugs.[5] As several studies show that chronic inflammation may be a critical component during carcinogenesis,[6] developing treatments against cancer-related inflammation is of great interest among many research groups at the moment.[7,8] Chronic pancreatitis is an inflammatory disorder often related to the development of pancreatic cancer.[9]

Terpenoids represent the largest group of natural products existing all over the plant kingdom, in microorganisms, fungi and marine sources where they play important roles in defending plants, animals and microorganisms against predators, herbivores and pathogens as well as delivering messages between different species.[10] Terpenoids are composed of isoprene units (C_5H_8) and classified into monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}) and tetraterpenoids (C_{40}). Extensive studies about the biological activies of terpenoids exist.[11–14] Currently, natural pentacyclic triterpenoids of oleanane-, ursane and friedelane-types are the most investigated compounds from this chemical class for their anticancer and anti-inflammatory activities.[15]

Abietanes or abietane-type diterpenoids with the characteristic carbon framework (Figure 1) are tricyclic diterpenoids that exist in the rosin of conifer trees and exhibit a wide range of biological activities.[16] Overall, like many natural products, most of them are not available in large amounts from their natural sources hampering the exploration of their medicinal potential. However, semisynthesis provides an efficient strategy to access more functionalized natural products using their simple building blocks as starting materials. Moreover, it enables the preparation of novel compounds in search of new drug leads and improved bioactivies. For instance, dehydroabietic (DAA, **1**) and abietic acids (AA, **2**, Figure 1) are commercially available and inexpensive naturally occurring abietanes, excellent starting materials for further functionalization.

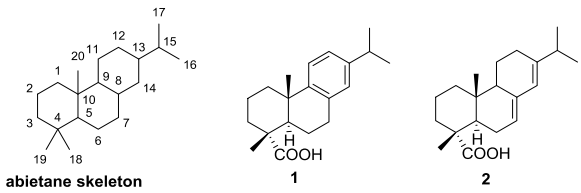


Figure 1 *Structures of dehydroabietic (**1**) and abietic acid (**2**) as well numbering of the abietane skeleton.*

Although AA is the most abundant resin acid, it is less stable than DAA because of the conjugated double bonds, thus easily degrading with light and temperature.[17] DAA belongs to aromatic abietanes which represent the majority of naturally occurring abietanes with around 200 characterized compounds.[16] DAA is reported to possess anticancer activities against several cancer types, and a number of semisynthetic derivatives have shown improved bioactivities.[18,19] Several biologically active aromatic abietanes contain oxygenated functionalities. Oxidation of benzylic C-H of aromatic abietanes is a key reaction to modify the abietane core. According to the literature, chromium(VI) reagents are most commonly used for this reaction.[20] Aromatic abietanes with a 13-propenyl side chain exist in some of scarcely available natural products such as angustanoic acid E and aquilarabietic acid H, both possessing interesting bioactivities *in vitro*.[21,22] Synthesis of this functionality is possible, for instance, from the corresponding 15-hydroxyl-dehydroabietanes via a dehydration reaction.

In the following literature review, the current situation related to the treatment of pancreatic cancer and the importance of natural products and their derivatives as potential anticancer agents are briefly covered. Furthermore, the relationship between cancer and inflammation and targeting cancer-related inflammation are discussed on a general level. Anticancer activities of selected naturally occurring abietanes as well as semisynthetic abietanes will also be discussed. In addition, synthesis methods for benzylic oxidation and 13-propenyl functionalization of dehydroabietanes used for the semisynthesis of known natural products are presented, including examples of their biological activities.

In the results and discussion section, the development of novel methods for benzylic oxidation of aromatic abietanes and bismuth(III) triflate-mediated dehydration of tertiary alcohols with a focus on providing environmentally sound and more sustainable options for the current methods among diterpenoids chemistry is presented. Moreover, the synthesis of novel dehydroabietic acid derivatives and studies about their biological activities against pancreatic cancer and inflammation is covered.

2 Review of the literature

2.1 Pancreatic cancer

Pancreatic cancer is the fourth leading cause of death by cancer in Europe and in the US.[23] In 2018, over 55000 new cases of pancreatic cancer will be diagnosed in the US.[3] The current treatment protocols for pancreatic cancer patients include surgery, with partial or total removal of the pancreas, radiation therapy, and chemotheraphy, depending on the type and stage of the diagnosed cancer. Nonetheless, the 5-year survival rates for this fatal disease still remain at 8% in the US and 3% in Europe, with most patients succumbing to the disease between 4.6 months and 2 years after diagnosis, clearly demonstrating the need to improve early diagnosis and to provide more effective and safer treatments.

Gemcitabine has served as the first line treatment for advanced pancreatic cancer for over two decades.[24] However, development of chemoresistance and difficulties of the drug to penetrate through the tumor stroma diminish its response rates.[25] Recently, FOLFIRINOX, a combination of oxaliplatin, irinotecan, fluorouracil, and leucovorin, as well as gemcitabine/nab-paclitaxel have replaced gemcitabine due to higher response rates and longer median overall survival achieved with these combination therapies.[26,27] However, the median survival was prolonged from 6.7 months in the gemcitabine to only 8.5 months in the gemcitabine/nab-paclitaxel group whereas with FOLFIRINOX the median survival was 11.1 months. Unfortunately, due to increased toxicity, FOLFIRINOX is suitable only for patients with a good performace status. Clinical trials for finding alternative improved therapies are also ongoing.[28]

Targeting pancreatic cancer remains challenging. According to a genetic analysis, 12 cellular signaling pathways and processes were altered in 67 to 100% of tumors containing on average 63 genetic mutations.[5] KRAS, TP53, CDKN2A, and SMAD4 are the four major driver genes in the disease development process, of which KRAS is the most dominant, as it is mutated in over 90% of tumors. Novel therapies[29] trying to target different elements in the development of pancreatic cancer include targeting signaling pathways involved in tumorigenesis, progression and metastasis, such as PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways mediaded by KRAS. Furthermore, therapies focusing on the tumor microenvironment, inflammatory response and angiogenesis are widely studied and will be discussed more detailed in the next chapter. Moreover, potential future therapeutic options could be cancer stem cell therapy or targeting epithelial-to-mesenchymal transition (EMT), microRNA or long noncoding RNAs (lncRNAs).

2.1.1 Cancer-related inflammation and its treatment strategies

Since Rudolf Virchow proposed for the first time, in 1863, the link between chronic inflammation and tumorigenesis,[30] numerous studies have focused on inflammation and cancer and the possibilities to use it as a prevention and/or treatment strategy. Although inflammation alone does not cause cancer, it is considered a critical component during

carcinogenesis.[6] According to epidemiological studies, at least 20% of all cancers initiate from a chronic inflammatory disease.[31] Oxidative stress is one of the features of chronic inflammation.[34] It is referred to as an imbalance between the production of free radicals and active intermediates and antioxidants neutralizing and eliminating them. Long-term oxidative stress may lead to chronic inflammation as reactive oxygen (ROS) or nitrogen species (RNS) can induce cellular damage and initiate inflammation.[35] Patients with chronic pancreatitis possess higher risk of developing pancreatic cancer.[36]

The tumor microenvironment (TME) and the inflammatory process can promote tumor initiation, promotion and metastasis. An increased production of growth factors, ROS and RNS that interact with the DNA of the proliferating cells induce malignant growth and tumor initiation in the surrounding tissue resulting in permanent genomic alterations. Multiple inflammatory mediators, including cytokines, chemokines, growth factors, free radicals, prostaglandins and proteolytic enzymes stimulate tumor development.[31] The major constituents of TME and the producers of these factors are macrophages, neutrophils, T cells, dendritic cells, natural killer cells, fibroblasts, adipocytes, and endothelial cells. Some of these factors can directly act on cancer cells while others act as protumorigenic factors affecting other components of the TME.[32]

The extracellular matrix (ECM) of the TME provides a physical scaffold for the cells promoting migration of both cancer and immune cells. ECM is important in cell signaling and contains key growth factors and chemokines that interact with cell surface receptors.[32,33] Tumor vasculature in the TME is different from normal blood vessels. It is heterogeneous and leaky which causes uneven blood flow, oxygenation as well as nutrient and drug distribution. Vascular endothelial growth factor (VEGF or VEGF-A) is the most dominant angiogenic factor in the TME promoting angiogenesis.

Tumor microenvironment offers several targets for the prevention and treatment of tumor-promoting diseases.[8] Anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAIDs) that primary target cyclooxygenase (COX) enzymes, which are overexpessed in several cancers, have shown potential in treating chronic inflammatory diseases and thus serving as effective chemopreventive agents. However, in long-term use they may cause several side effects such as renal failure and gastrointestinal (GI) symptoms, potentially increasing cancer risk.[31]

According to several preclinical studies, statins, namely 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, were able to inhibit cancer cell proliferation, induce apoptosis and decrease tumor growth as well as inhibit angiogenesis.[37] Anti-inflammatory steroids, on the other hand, serve as supportive medication in cancer-related cachexia. Cytokines and chemokines, such as TNF-α, interleukin-6 (IL6), CCL2, TGF-β, interleukin-1α, interleukin-8 and CXCR4, produced by both normal (tumor supporting) and tumor cells, act as molecular messengers and contribute to several hallmarks of cancer. From these mediators, TNF-α and IL-6 are two major tumor-promoting cytokines, widely studied as drug targets against several cancers.[8]

Furthermore, the JAK/STAT pathway is involved in the regulation of cell proliferation and survival, and JAK and STAT inhibitors represent promising targets for treating cancer-related inflammation. Especially STAT3 has shown to be an important mediator in the pathogenesis of pancreatic cancer.[38] Another important target is the transcription factor nuclear factor-κB (NF-κB) pathway which, once activated, increases the expression of

genes involved in cell survival and proliferation. NF-κB is activated in early stages of pancreatitis and prolonged active stage often leads to the development of chronic pancreatitis and finally pancreatic cancer.[39,40]

Tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) highly promote cancer-related inflammation.[8] The chemotherapeutic agent trabectedin has myeloid-cell targeting properties and is licensed for use in patients with resistant soft-tissue sarcoma and ovarian cancer. Several agents targeting TAMs are in clinical trials.[41] Targeting the receptor of colony-stimulating factor 1 CSF-1R has shown to eliminate TAMs in several tumor types *in vitro* and *in vivo*.[31]

Synthetic oleanane triterpenoids (SO) have profound effects on inflammation and oxidative stress and are widely studied as multifunctional drugs highly effective for the prevention and treatment of cancer according to several animal models. The oleanolic acid derivatives methyl 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) and CDDO-imidazolide (CDDO-Im) suppress the production of the inflammatory mediator inducible nitric oxide synthase and inhibit the production of IL-1β, IL-6, TNFα, VEGF and CCL2 in both immune and cancer cells.[42,43] They also reduce oxidative stress by activating the Keap1/Nrf2/ARE signalling pathway, involved in the regulation of the transcription of many antioxidant genes. DAA also shows activity as a peroxisome proliferator-activated receptor γ (PPAR-γ) agonist and suppresses the production of pro-inflammatory mediators, such as MCP-1, TNF-α and nitric oxide (NO) making it potentially relevant for the treatment of cancer-related inflammation.[44,45]

2.2 Natural products and cancer

2.2.1 Nature-derived anticancer drugs

Natural based anticancer drugs are typically isolated from terrestrial origin including different parts of plants and micro-organisms as well as from marine sources.[46,47] Some of the most well-known plant-based anticancer drugs are vinca alkaloids vinblastine and vincristine isolated from the Madagascar periwinkle, *Catharanthus roseus* (L.) G.Don.[48] as well as paclitaxel (Taxol®) existing in the bark of the Pacific yew tree, *Taxus brevifolia* Nutt., and its analog docetaxel.[49] Nowadays, paclitaxel and its derivatives are produced semisynthetically from the needles of another Pacific yew tree, *Taxus baccata* L. Moreover, etoposide and teniposide, derived from epipodophyllotoxin, isolated from the roots of *Podophyllum* species,[50] as well as campthothecin, isolated from the bark of *Camptotheca acuminate* Decne., and its derivatives topotecan (Hycamptin®) and irinotecan (Camptosar®) are all well-known plant-based anticancer drugs.[51–53]

Lately, the increased interest towards studying marine natural products has led to the discovery of new structurally diverse compounds from different chemical classes, including a variety of alkaloids, amine derivatives, macrolides, peptides/polypeptides, phenols/polyphenols, polysaccharides, quinones, steroids and terpenes possessing several anticancer activities.[54] Marine sponges are commonly rich sources of new natural

products.[55] To date, eight marine-derived drugs have been approved for clinical use, of which five are anticancer drugs.[56–58] In addition, several others are currently in clinical trials.

Cytarabine or Ara-C (Cytosar-U®), an analog of nucleosides isolated from the Caribbean sponge *Tectitethya crypta* (de Laubenfels, 1949) was approved in 1969 for the treatment of leukemia and lymphoma. Trabectedin or ecteinascidin 743 (ET-743, Yondelis®) is a marine tetrahydroisoquinoline alkaloid produced by the tunicate *Ecteinascidia turbinate* (Herdman, 1880) approved in 2007 for advanced or metastatic soft tissue carcinoma and in 2009 for ovarian cancer therapy in combination with pegylated liposomal doxorubicin DOXIL®/Caelyx®. Eribulin mesylate (E7389, Halaven®), a simplified analog of a natural macrocyclic polyether halichondrin B, existing in several species of marine sponges, is used for the treatment of metastatic breast cancer. Monomethyl auristatin E, used as an antibody-drug conjugate called brentuximab vedotin, was accepted in 2011 for the treatment of lymphoma. The analgesic cone snail-derived peptide ziconotide (Prialt®) is according to some reports classified as an anticancer drug as it is used to relieve cancer-related pain.[59]

Several nature-derived anticancer drugs in the market are antibiotics produced by microbes. These include multiple anthracyclines, such as daunorubicin, doxorubicin (adriamycin), epirubicin, pirarubicin, idarubicin, valrubicin, amrubicin and actinomycin D as well as glycopeptides (bleomycin, phleomycin), mitomycin C, and the anthracenones (mithramycin, streptozotocin, pentostatin).[60]

Overall, the chemical diversity and the unique chemical space occupied by natural products is astonishing. Nature provides us with complex structures, novel scaffolds and chemical entities that would require enormous efforts if produced synthetically. However, one of the major difficulties the natural product research is facing is the difficult and expensive isolation process which usually produces only minor amounts of pure compounds. It is also often argued that natural products are not drug-like as they do not normally follow the so-called Lipinski rule of five to be orally active drugs. However, a study shows that natural product leads had an identical success rate (50%) in delivering an orally available drug than drugs following the Lipinski rules.[61] Often, semisynthesis offers a way to improve the binding affinities and druglike properties of natural products making them more bioavailable.

2.2.2 Abietane-type diterpenoids and cancer

Naturally occurring abietanes with anticancer activity

Several abietanes isolated from natural sources possess interesting biological activities. A few of the most widely studied anticancer agents from this compound class are shown in Figure 2. Some further examples will be presented later while oxygenated aromatic abietanes and their biological activities are discussed. Tanshinone IIA (**3**), one of the major constituents in *Salvia miltiorrhiza* Bunge has shown to induce apoptosis and inhibit pancreatic cancer cell proliferation *in vitro*,[62,63] as well as induce endoplasmic reticulum (ER) stress in BxPC-3-derived xenograft tumors *in vivo*.[64] Moreover, it has also shown

activity against several other cancer types.[65,66] Aromatic abietanes carnosol (**4**) and carnosic acid (**5**) existing in the leaves of *Salvia officinalis* L. (sage) have also shown versatile activities against several cancer lines both *in vitro* and *in vivo*.[67–69] In addition, they possess anti-inflammatory activity and have shown to interfere with multiplte signalling pathways which are deregulated during inflammation and cancer.[70]

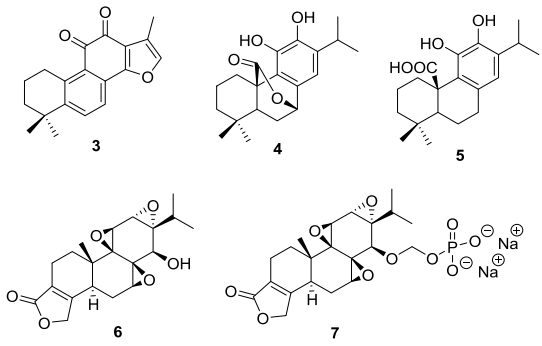


Figure 2 *Structures of tanshinone IIa (**3**), carnosol (**4**), carnosic acid (**5**), triptolide (**6**) and minnelide (**7**) studied against several cancer types.*

Triptolide (**6**) isolated from the *Tripterygium wilfordii* Hook. f.[71] exhibits a wide range of anticancer properties and according to a number of preclinical studies it inhibits, for instance, cancer cell proliferation and metastasis as well as induces apoptosis.[72,73] Interference with multiple pathways are recognized as potential mechanisms of action, however, no clear target has yet been identified. Poor water solubility hampers the further development of triptolide and attempts to improve its PK properties led to synthesis of new derivatives and to the discovery of minnelide (**7**), a prodrug of triptolide with a phosphate group attached to the free hydroxyl group of triptolide making it more water-soluble. Once minnelide has reached the bloodstream, triptolide is released. Minnelide has shown preclinical activity against pancreatic cancer[74] and patients are currently being recruited for a phase II clinical trial[75] in refractory pancreatic cancer. A phase I clinical trial in patients with relapsed or refractory acute myeloid leukemia[76] is currently on hold whereas a phase I clinical trial in advanced gastrointestinal tumors[77-78] has recently ended and progression onto phase II is pending. Furthermore, triptolide has been a compound of interest for preparing further semisynthetic derivatives aiming at improved bioavailability, anticancer properties and understanding structure-activity relationships (SAR).[79–82]

Semisynthetic abietanes with anticancer activity

The interest towards developing novel semisynthetic abietanes with improved bioactivities is constantly increasing as several research groups aim at finding novel anticancer agents using for instance DAA, AA and dehydroabietyl amine as starting materials, of which DAA represents by far the most popular. Two independent studies reported semisynthesis of novel

DAA derivatives where positions 7 and 18 both have been functionalized. In the first study, novel chiral dipeptide derivatives were evaluated for their antiproliferative activity against human epithelial cervical (HeLa), lung (NCI-H460) and gastric (MGC-803) cancer cell lines studied by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[83] In addition to the dipeptide moiety, the compounds beared a carbonyl or an oxime group at position 7. The most active compounds of the study are included into Figure 3.

Compounds **8**, **9** and **10** showed markedly improved antitumor activity against HeLa cancer cell line compared to DAA (IC_{50} = 29.35 μM) and the anticancer drug 5-fluorouracil (5-FU, IC_{50} = 36.53 μM). Compound **8** had the best activity against the MGC-803 cancer cell line. None of the compounds had very good activity against lung cancer cells except compound **11**. However, all compounds were more active against all the cancer cell lines than DAA or 5-FU. Compounds also had low cytotoxicity on normal human liver cell lines (HL-7702), indicating good selectivity. SAR observations revealed that the presence of an L-configuration favored *ortho*-substitution (compound L-**8** and L-**10**) whereas *para*-substitution was favored by D-configuration (D-**11**). Compound **8** was the most potent against HeLa cells and induced apoptosis most likely through mitochondrial apoptotic pathways.

In a second study, the DAA derivatives were also functionalized with a carbonyl or an oxime group at position 7 but the dipeptide moiety was replaced with a thiourea α-aminophosphonate group.[84] Herein, the antiproliferative activity of the compounds was studied against human NCI-H460, lung adenocarcinoma (A549), liver (HepG2) and ovarian (SKOV-3) cancer cell lines. Overall, most compounds showed better activity than DAA (IC_{50} = 79.46-85.00 μM) and 5-FU (IC_{50} = 24.43-44.04 μM) as shown in Figure 3. Compounds **12**, **13** and **14** inhibited the proliferation of SKOV-3 cells but only **13** was active against NCI H460 cells. Compounds **15-17** were the most potent against HepG2 cells and whereas compounds **14**, **15**, **17** and **18** were the most potent against A549 cells.

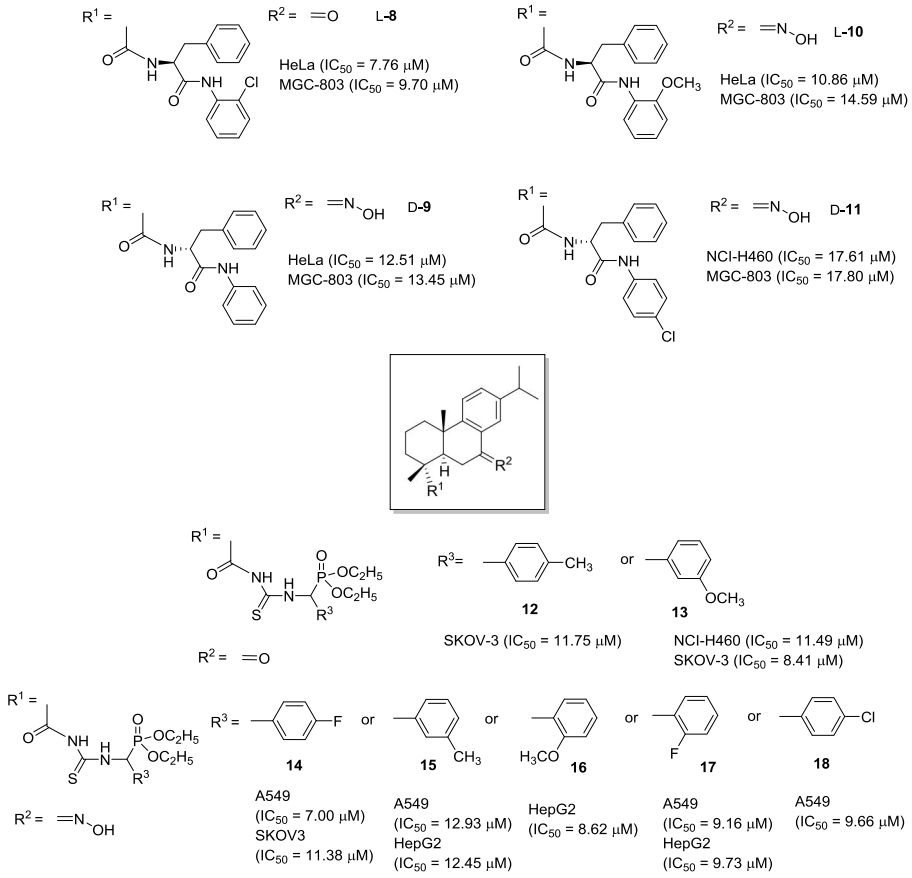


Figure 3 *Dipeptide and thiourea α–aminophosphonate DAA derivatives and their antiproliferative activities against NCI-H460, HeLa, MGC-803, SKOV-3, HepG2 and A549 human cancer cell lines.*

Further thiourea derivatives were examined in other studies. Huang *et al.* explored the effects of substituting position 18 with thiourea derivatives containing a bisphosphonate moiety (Figure 4).[85] The cytotoxicities of these compounds were evaluated *in vitro* against human SKOV-3, hepatoma (BEL-7404), A549, breast carcinoma (HCT-116) and NCI-H460 cell lines. The most active compound against all the cell lines was **19**, the only compound with a fluorine substituent in position 4 of the phenyl ring, having an IC_{50} value below 10 μM. For DAA, the IC_{50} values varied from 28.72 to 75.05 μM. Compared to the thiourea α-aminophosphonate derivatives presented previously, the activities were rather similar. The only clear difference was in the ovarian cancer cell line where **19** had an IC_{50} of 1.79 μM compared to **13** (IC_{50} = 8.41 μM) and **14** (IC_{50} = 11.38 μM).

Xing *et al.* synthesized a set of 18-functionalized DAA derivatives functionalized via a thiourea linker as well. The most active compound was the quinidine derivative **20** (Figure 4) which was active against bladder (EJ and 5637), prostate (PC-3), HeLa and leukemia (Jurkat) cell lines, with IC_{50} as depicted on Figure 4. Mechanistic studies revealed that **20**

along with two other thiourea derivatives, induced apoptosis in EJ cells mainly through a mitochondrial-dependent pathway, with increased levels of cytochrome c release and downregulation of the expression of procaspase-9 and procaspase-3. Compound **20** was also the only thiourea derivative that had no significant toxicity towards normal cells.[18]

In another *in vitro* screening of thiourea substituted DAA derivatives, the acylthiourea **21** (Figure 4) showed the best activity against A549 and liver (SMMC7721) cancer cell lines with an IC_{50} of 6.44 and 6.84 μM, respectively.[86]

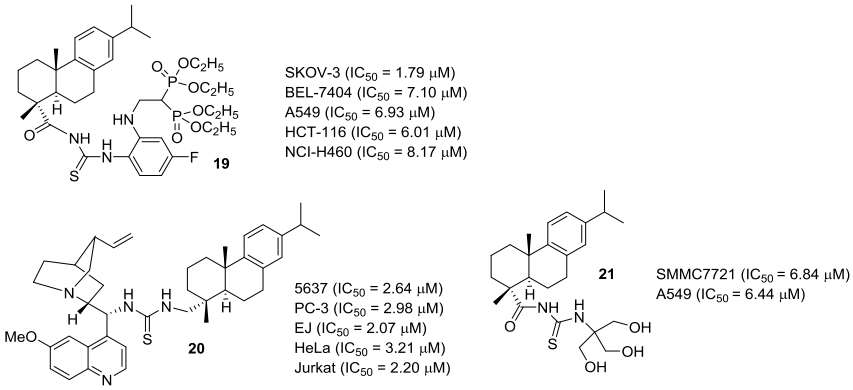


Figure 4 *Thiourea DAA derivatives **19-21** with anticancer activity against various human cancer cell lines.*

The screening of a series of unsymmetrically *N*,*N*'-substituted urea derivatives of DAA revealed four active compounds, **22-25** against the SMMC7721 cell line (Figure 5).[87] The unsubstituted urea compound **22** had the best antiproliferative activity indicating that improved activities could possibly be searched by modifying other positions of the abietane core since substitution of the urea with larger substituents actually slightly diminished the activity.

Compounds bearing an acylhydrazone moiety were synthesized from DAA and evaluated for their antiproliferative activities against human CNE-2 (nasopharynx), HepG2, HeLa, and liver (BEL-7402) cancer cell lines by MTT assay *in vitro*.[88] The most potent compounds **26-30** (Figure 5) had IC_{50} values ranging from 2.21 to 11.45 μM which were significantly better than for DAA (37.40-88.64 μM). The presence of fluorine was found to be important for the activity as **30** was the only fluorine-containing derivative active below 10 μM. It was especially potent towards HeLa cells with an IC_{50} of 2.21 μM.

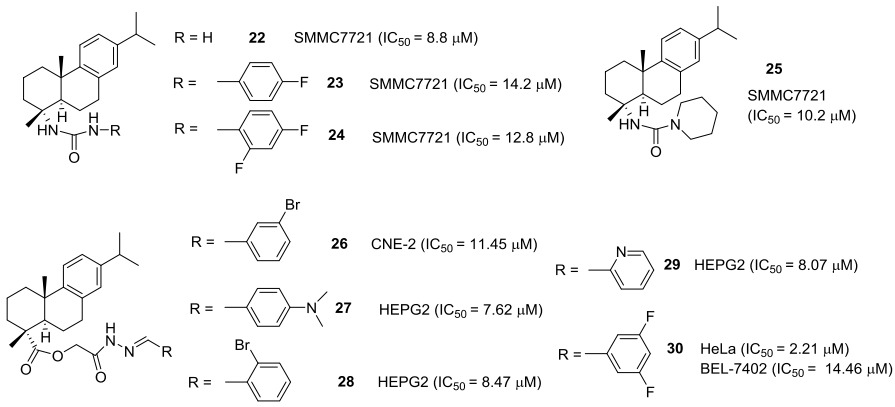


Figure 5 *Urea and acylhydrazone substituted DAA derivatives and their IC_{50} values towards various human cancer cell lines.*

Two independent studies evaluated triazole derivatives of DAA as novel anticancer agents. The 1,2,3-triazole moiety was introduced to the relatively unexplored position 14 of DAA to create a novel 1,2,3-triazole dehydroabietic hybrid scaffold.[89] Compounds were screened *in vitro* in SKOV-3, PC-3 and breast (MDA-MB-231 and MCF-7) cancer cell lines by MTT assay. The most potent compounds **31**-**34** (Figure 6) had IC_{50} values ranging from 0.7 to 2.3 μM. Moreover, evaluation of the intermediates suggested that the 1,2,3-triazole moiety was essential for the activity. SAR-examination further revealed that introduction of electron-rich aromatic ring systems such as methoxy significantly improved activity. The compounds also mostly followed the Lipinski rule of five concerning LogP, topological polar surface area (TPSA) and the number of hydrogen bond donors and acceptors.

Pertino *et al.* designed and synthesized a set of compounds with a triazole group at position 18 attached via dehydroabietyl alcohol and studied their anticancer activities against lung fibroblasts (MRC-5), gastric epithelial adenocarcinoma (AGS), lung cancer (SK-MES-1) and bladder carcinoma (J82) cell lines. Only compound **35** (Figure 6) showed potency in SK-MES-1 similar to that of the known anticancer agent etoposide (1.83 μM).[90]

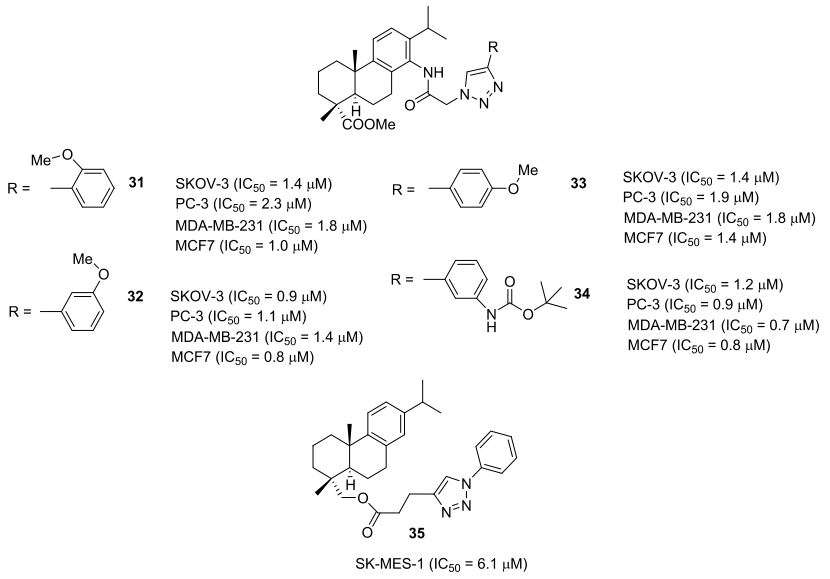


Figure 6 *Triazole-substituted DAA derivatives and their anticancer properties.*

Ukiya *et al.* evaluated a set of amino acid derivatives of AA and DAA against human HL60, A549, stomach (AZ521), and breast (SK-BR-3) cancer cell lines. Several compounds (**36-44**) showed improved potency towards HL60 cells compared to AA and DAA (AA, IC_{50} = >100 μM and DAA, IC_{50} = 66.7 μM) as depicted on Figure 7. Only compound **44** was active against all the cell lines.[91]

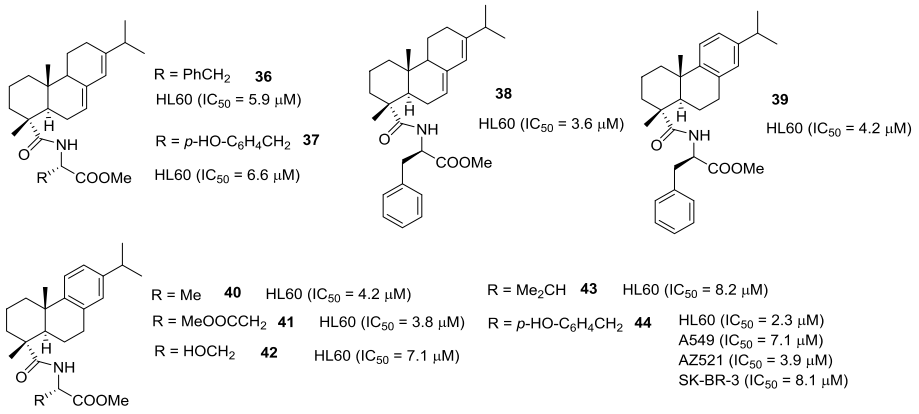


Figure 7 *Amino acid derivatives of DAA and AA with anticancer activity.*

Finally, DAA dipeptide derivatives containing a sulfonamide moiety were synthesized and evaluated for the inhibition of matrix metalloproteinases (MMPs) MMP-3, MMP-8 and MMP-9 as well as for their antiproliferative activities and ability to affect cell migration *in*

vitro.[92] Positions 18 and 12 were functionalized with substituted phenylalanines of which one contained a sulfonamide. Most compounds were found to preferentially inhibit MMP-3, the best compounds being **45**-**47** (Figure 8). Inhibition towards other MMPs was weaker, however, compound **46** was the most potent, with an IC_{50} value of 6.6 μM for MMP-8 and 8.8 μM for MMP-9, respectively. Methyl groups as substituents in the aromatic ring enhanced MMP-3 inhibition. Compounds **46** and **47** showed antiproliferative activity against NCI-H46, HepG2, SKOV-3 and MCF-7 tumor cell lines with IC_{50} values ranging from 4.2 to 13.1 μM, whereas **45** was only active against HEPG2 cells. Compound **46** was found to inhibit *in vitro* cell migration, cause cell cycle arrest in G1 phase as well as to induce apoptosis in HEPG2 cells, thus being a potent MMPs inhibitor for further development.

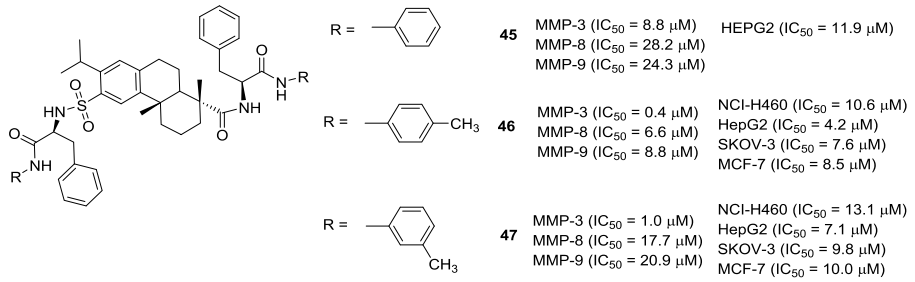


Figure 8 *DAA dipeptide sulfonamide derivatives with MMP inhibition activity.*

2.3 Target synthetic methods for derivatization of the abietane core

2.3.1 Benzylic oxidation of aromatic abietanes

Aromatic abietanes have two possible positions for benzylic oxidation to occur, namely positions 7 and 15 of the abietane core, depending on the reagents and reaction conditions used. The different methods reported in the literature for the benzylic oxidation of aromatic abietanes will be discussed in this section.

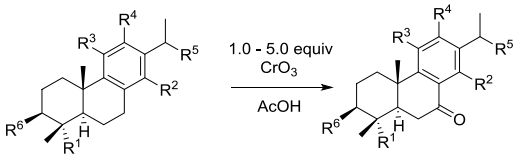
Chromium(VI) reagents

Chromium(VI) reagents are the most widely used reagents for the benzylic oxidation of aromatic abietanes at position 7. Chromium(VI) oxidation is commonly one of the synthesis steps to modify the abietane core to produce novel diterpenoids with various biological activities as well as for the semisynthesis of naturally occurring diterpenoids. Chromium exists at oxidation states from –II to +VI, -III and +VI being the most common ones.[93] Cr(III) reagents have generally low toxicity, whereas chromium(VI) reagents are highly

toxic and environmentally hazardous.[94] However, chromium is a strong oxidant, has good functional group tolerability and gives the 7-oxo derivatives as major products with good selectivity, which is why most scientists still rely on it. Moreover, so far not many alternatives to replace chromium exist.

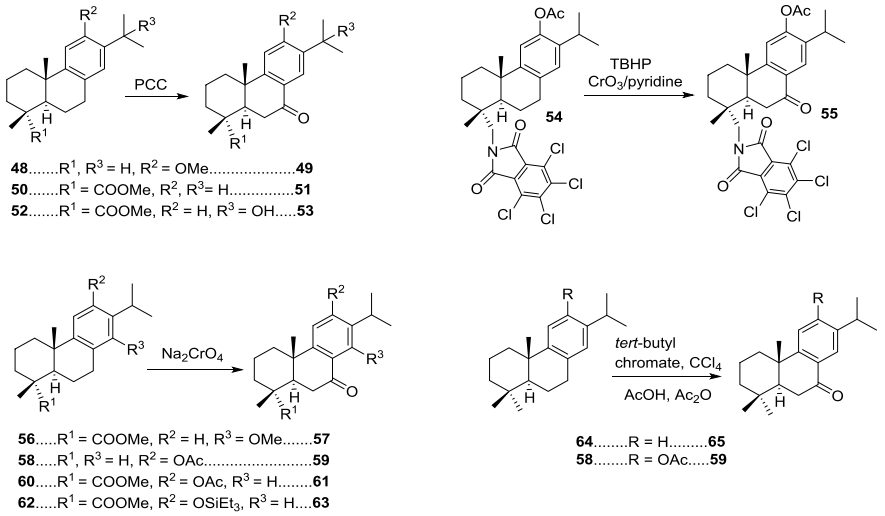
To illustrate the versatile use of chromium and its applicability for differently substituted aromatic abietanes, Table 1 and Scheme 1 summarize most of chromium reactions performed according to the literature. Table 1 provides examples of the oxidation reactions in acetic acid, mediated by chromium(VI) trioxide (CrO_3), the most common chromium reagent. In many cases, acetic anhydride is also included in the reaction mixture to improve the solubility of CrO_3. Other common reagents for benzylic oxidation of aromatic abietanes are pyridinium chlorochromate (PCC),[95,96] sodium chromate (Na_2CrO_4),[97–100] *tert*-butyl chromate[101] as well as CrO_3 as a catalyst with pyridine in excess of *tert*-butyl hydroperoxide (TBHP)[102] (Scheme 1). In acidic media, CrO_3 and Na_2CrO_4 form chromic acid *in situ* which is responsible for the actual oxidation of the reactant.

Table 1 *CrO_3-mediated oxidations reported in the literature.*



Entry	R	Reference	Entry	R	Reference
1	R^1 = COOMe R^2, R^3, R^4, R^5, R^6 = H	Ritchie *et al.*[103] Monteiro *et al.*[104] Gu *et al.*[105,106]	**8**	R^1 = CH_2OAc R^4 = OAc R^2, R^3, R^5, R^6 = H	Alvarez-Manzaneda *et al.*[107]
2	R^3 = OAc R^4 = OBz R^1, R^2, R^5, R^6 = H	Yang *et al.*[43]	**9**	R^1 = COOH R^2, R^3, R^4, R^5, R^6 = H	Huang *et al.*[83]
3	R^4 = OMe R^1, R^2, R^3, R^5, R^6 = H	Li *et al.*[108]	**10**	R^4 = OMe R^1, R^2, R^3, R^5, R^6 = H	Córdova-Guerrero *et al.*[109]
4	R^3 = OAc R^4 = OCOPh R^1, R^2, R^5, R^6 = H	Matsumoto *et al.*[110]	**11**	R^2, R^3= OAc R^1, R^4 = H	Matsushita *et al.*[111]
5	R^1 = COOMe R^2, R^4 = Cl R^3, R^5, R^6 = H	Cui *et al.*[112]	**12**	R^1 = COOMe R^2, R^3 = OAc R^4, R^5, R^6 = H	Matsushita *et al.*[111]
6	R^1 = COOH R^2, R^4 = Cl R^3, R^5, R^6 = H	Ohwada *et al.*[113]	**13**	R^1 = COOMe R^2, R^3= OAc R^4, R^6 = H R^5 = CH_2OAc	Matsumoto *et al.*[114]
7	R^1 = CH_2OTs R^4 = OBn R^2, R^3, R^5, R^6 = H	Alvarez-Manzaneda *et al.*[107]	**14**	R^3= OMe R^1, R^2, R^4, R^5 = H R^6 = OAc	Burnell *et al.*[115]

As shown here, CrO_3 allows the oxidation of dehydroabietanes bearing different functional groups. Many of these compounds are used as intermediates in the synthesis of known natural products or novel compounds for chemical libraries and bioactivity screenings. Typical yields reported for these oxidations vary from 50 to 80% and reaction time from 0.5 to 24 hours. The amounts of CrO_3 used range from 1.0 to 5.0 equiv. Heating is used in many cases, although some studies also report the use of 0 °C temperature at the beginning of the reaction after which it is raised to room temperature.



Scheme 1 *PCC, Na_2CrO_4, tert-butyl chromate and TBHP in combination with catalytic CrO_3 /pyridine mediated benzylic oxidations of aromatic abietanes.*

The other chromium(VI) reagents work also very well for dehydroabietanes with various functionalities, however, they are clearly less often used as CrO_3. Reaction conditions and yields do not differ much from CrO_3. Reported yields are slightly higher, ranging from 50 to 95% and room temperature or heating under reflux are generally used. Most of the studies do not report the amounts of chromium(VI) reagent used but in the available ones it varies from 2.0 to 3.0 equiv apart from the one where catalytic amounts of CrO_3 are used.

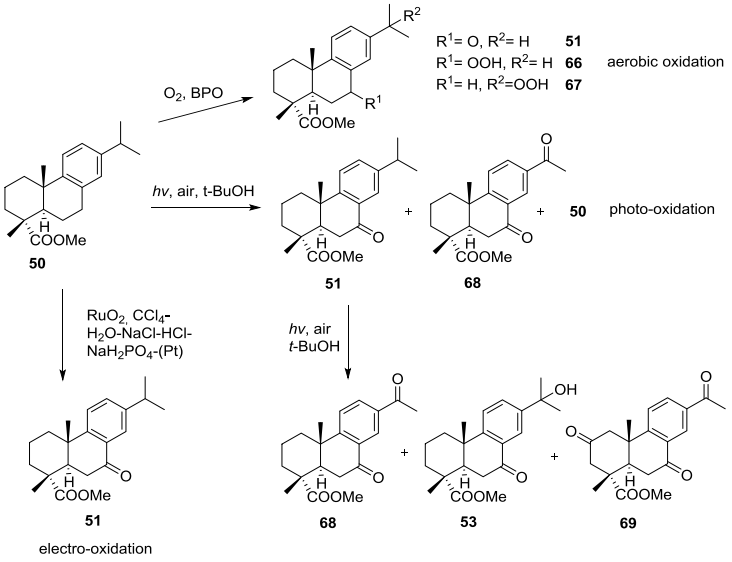
Other oxidants for benzylic oxidation

Other oxidation methods include several metal-based reagents with and without the use of molecular oxygen as well as photo-oxidation and electro-oxidation. Most of these methods produce several oxidation products where typically both benzylic positions of aromatic abietanes are oxidized.

Ritchie *et al.*[116] performed the first aerobic oxidations of aromatic abietanes with molecular oxygen in combination with dibenzoyl peroxide (BPO) in the early 1950's. Oxidation of methyl dehydroabietate **50** with molecular oxygen and 2.4 mol % of BPO at

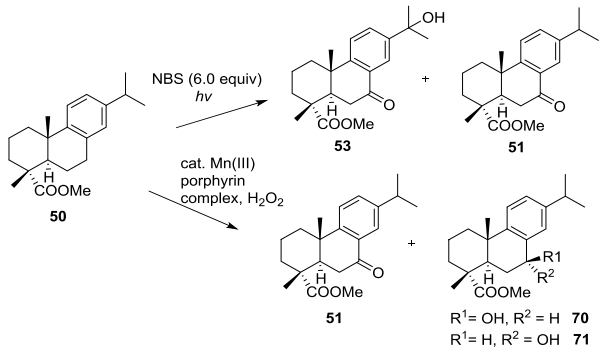
80 °C produced, after 43 hours, the 7-oxo **51** and methyl 7-hydroperoxy **66**, in 25% and 29% yields, respectively, together with a small amount of the 15-hydroperoxy **67** product (Scheme 2).

Photo-oxidation of **50** in 0.2 M *t*-BuOH under irradiation of a mercury vapor lamp with exposure to air produced, after 38 hours, **51** and the 13-acetyl **68** in 37% and 1% yields, respectively, as well as 47% of the starting material (Scheme 2). Irradiation of **51** under similar conditions for 73 hours yielded 30% of **68**, 21% of the 7-oxo-15-hydroxy product **53** and 12% of the diketone product **69** which structure was confirmed by X-ray crystallography.[117] Ruthenium tetroxide-mediated electro-oxidation of **50** produced **51** in 52% yield (Scheme 2).[118]



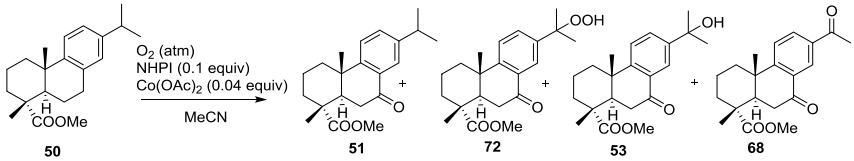
Scheme 2 *Products obtained in benzylic aerobic, photo- and electro-oxidations of methyl dehydroabietate **50**.*

Benzylic oxidation of **50** with *N*-bromosuccinimide[119] (NBS) under irradiation of light gave the **53** derivative as the major product, in 79% yield, whereas only minor amounts of **51** formed (Scheme 3). Similar products were obtained with other starting materials differing only from the functional group at position 18. Cavaleiro *et al.* studied manganese(III) porphyrin complexes as catalysts in combination with H_2O_2 for the benzylic oxidation of **50** with poor outcomes (Scheme 3). The highest yield obtained for **51** was 11%.[120]



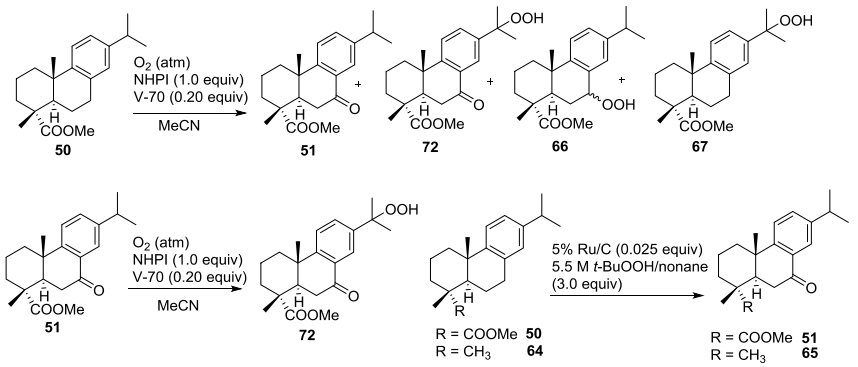
Scheme 3 *N-bromosuccinimide (NBS) and manganese(III) porphyrin complex-based benzylic oxidations.*

Matsushita *et al.*[121] studied the aerobic oxidation of **50** under milder conditions using *N*-hydroxyphthalimide (NHPI) in combination with either cobalt(II) acetate or 2,2-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) as co-catalyst systems. Treatment of **50** under oxygen (1 atm), in acetonitrile, in the presence of 0.1 equiv of NHPI and 0.04 equiv of cobalt(II) acetate, at 30 °C, for 48 hours, gave **51**, **72**, **53**, and **68** in 6%, 24%, 44%, and 14% yields, respectively (Scheme 4). When the temperature was raised to 50 °C, after 48 hours, only **53** and **68** formed, in 55% and 20% yields, respectively. On the other hand, when the reaction time was shortened to 24 hours at 30 °C, **51** and **72** formed, in 40% and 38% yields, together with 13% of **53**, and only residues of **68**. Similar results were obtained after 48 hours when the temperature was lowered to 23 °C.



Scheme 4 *Aerobic benzylic oxidation of methyl dehydroabietate and methyl 7-oxo-dehydroabietate with Co(II) acetate in combination with NHPI.*

Aerobic benzylic oxidation with V-70 in combination with NHPI as the catalyst under oxygen (1 atm), in acetonitrile, produced compounds **51**, **66**, **67** and **72**, in 16%, 8%, 10% and 41% yields, respectively (Scheme 5). A single product **72** formed in 90% yield under same conditions when **51** was used as the starting material. The amounts of one equiv of NHPI and 0.20 equiv of V-70 were necessary to exhaust all the starting material. Finally, Matsushita *et al.* studied the oxidation of **50** and **64** with 5% Ru/C in ethyl acetate in combination with *t*-BuOOH which produced **51** and **65**, in 70% and 78% yields, respectively (Scheme 5).



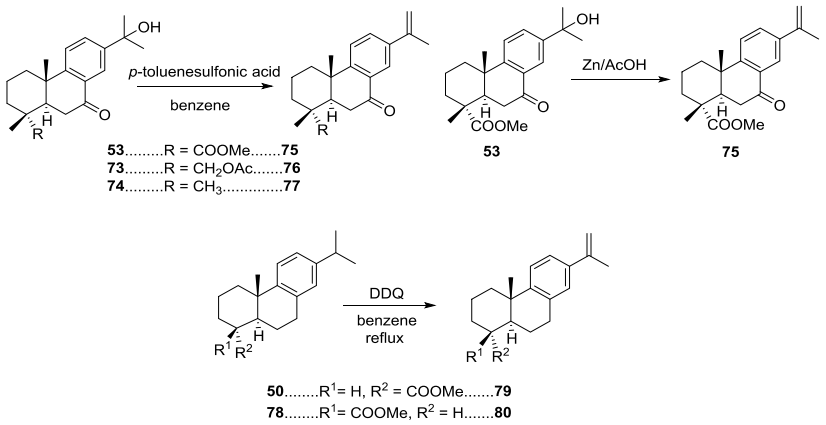
Scheme 5 *Aerobic benzylic oxidation of methyl dehydroabietate and methyl 7-oxo-dehydroabietate with V-70 in combination with NHPI.*

The benzylic oxidation of aromatic abietanes is possible with aerobic, photo- and electro-oxidation methods including several metal-based reagents. Most of these methods suffer from disadvantages such as the need for expensive speciality reagents, possible end product contamination with trace metallic impurities as well as the production of large amounts of metal waste. Moreover, compared to chromium, yields are lower and mixtures of products form in most cases as both benzylic positions are oxidized.

2.3.2 Incorporation of 13-propenyl group onto aromatic abietanes

A 13-propenyl group is common to several natural products including antiochic acid,[122] diterpenoids from *Pinus massoniana* Lamb,[123] aquilarabietic acid H[21] from Chinese eaglewood (*Aquilaria sinensis* Gilg) as well as bodinieric acids C and F from *Callicarpa bodinieri* H.Lév.[124] According to literature this functionality is usually built from 15-hydroxy dehydroabietanes or from methyl dehydroabietate **50**, however, not many studies exist where this transformation is reported.

The available methods for the preparation of 13-propenyl abietanes are shown in Scheme 6. The dehydration of 7-oxo-15-hydroxy dehydroabietanes **53**, **73** and **74** with traces of *p*-toluenesulfonic acid in refluxing benzene gave alkenes **75**, **76** and **77**.[119] Zn in acetic acid, on the other hand, was able to dehydrate compound **53** producing the alkene **75**.[125] Moreover, compounds **50** or **78** were reduced to the alkenes **79** and **80** using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in refluxing benzene. This synthesis is reported by several research groups with yields varying between 45-70%.[126–128]



Scheme 6 *Reported methods for synthesizing 13-propenyl-functionalized aromatic abietanes.*

In two of these reactions hazardous benzene is used as a reaction solvent. In addition, they require excess of reagents or metals in strongly acidic solutions, both disadvantageous, for instance, in total synthesis routes.

2.4 Semisynthesis and biological activities of relevant abietanes

Oxygenated and 13-propenyl-functionalized aromatic abietanes exist widely in nature and possess a variety of biological activities. In this chapter, a few semisyntheses of known natural products, where the synthetic scheme includes a benzylic oxidation step or a 13-propenyl intermediate, are presented. The examples selected here highlight the importance of benzylic oxidation in different synthetic routes towards naturally occuring aromatic abietanes.

2.4.1 Biological activities of naturally occuring oxygenated and 13-propenyl aromatic abietanes

Naturally occurring oxygenated aromatic abietanes possess a variety of biological activities. Herein, the bioactivities of twelve selected compounds (Figure 9) are presented. Sugiol (**81**),[129] isolated from the tree *Juniperus polycarpos* K.Koch, showed weak antimalarial activity against three *Plasmodium falciparum* clones D6, TM91C235 and W2.[130] Furthermore, **81**, extracted from the conifer *Cryptomeria japonica* (Thunb. ex L.f.) D.Don, exhibited anti-inflammatory activity,[131] and **81** isolated from the roots of *Peltodon longipes* A.St.-Hil. ex Benth showed moderate anticancer activity in human pancreatic (MIAPaCa-2) and melanoma (MV-3) tumor cell lines *in vitro*.[63] In addition, **81**, extracted from *Amentotaxus formosana* H.L.Li, inhibited xanthine oxidase (XO) activity and ROS formation.[132] In addition, some studies have reported moderate antimicrobial activities for **81**.[133,134]

Galdosol (**82**) existing in the leaves of sage *Salvia officinalis L.* exhibited antioxidant activity[135] as well as strong binding to the benzodiazepine receptor.[136] Salvinolone (**83**), extracted from the roots of *Salvia prionitis* Hance,[137] and demethylcryptojaponol (**84**), existing in the root of *Salvia phlomoides* Asso,[138] showed potent antimicrobial activity against three MRSA strains as well as against two VRE strains.[139] Moreover, hanagokenol A (**85**) isolated from *Cladonia rangiferina* (L.) Weber ex F.H.Wigg. (1780) was found to exhibit moderate antimicrobial activity.[133]

The oxygenated abietanes 7-oxo-callitrisic acid (**86**) and 15-hydroxy-7-oxo-dehydroabietic acid (**87**), existing in the aerial parts of *Abies georgei* Hand.-Mazz., were studied for their anti-inflammatory and cytotoxic activities. Compound **86** was more active against human hepatocellular carcinoma QGY-7703 cells.[140] In addition, another study reported that 7-oxo-dehydroabietic acid with reversed stereochemistry in carbon 4 compared to **86** exhibited induced neuroprotective activity at 20 μM measured by nerve growth factor (NGF) secretion in glioma C6 cells. The secretion was about 200% whereas the positive control, 6-shogaol induced the secretion with only about 140% (untreated control was set to 100%). Furthermore, no cytotoxic effects occurred at the tested concentration.[141]

7α,15-Dihydroxydehydroabietic acid (**88**) isolated from a water extract of *Pinus koraiensis* Siebold & Zucc. cones exhibited anti-angiogenic activity measured in human umbilical vein endothelial cells (HUVECs). Inhibition occurred through downregulation of VEGF, p-Akt and p-ERK protein levels.[142] Interestingly, the 4-epi-7α,15-dihydroxydehydroabietic acid (**89**) isolated from the stems of *Illicium jiadifengpi* B.N. Chang, having reversed stereochemistry in carbon 4 compared to **88**, showed moderate antiviral activity against Coxsackie virus B3 (CVB3).[143] Angustanoic acid F (**90**), existing in the roots of *Illicium jiadifengpi*, was also evaluated *in vitro* for its antiviral activity against Coxsackie viruses B2, B3, B4 and B6. Most potent activity was observed against B2 and B6 viruses, however, the selectivity index values remained relatively low compared to **89**.

Coleon U (**91**) isolated from *Plectranthus grandidentatus* Gürke and 14-deoxycoleon U (**92**) isolated from *Salvia leriifolia* Benth. exhibited both promising antiproliferative activities against several cancer cell lines. Compound **91** was active against five human tumor cell lines, namely MCF-7, NCI-H460, central nervous system cancer (SF-268), renal cancer (TK-10) and melanoma (UACC-62). Compound **92**, on the other hand, had antiproliferative activity against PC-3 and HeLa cancer cell lines, and it was additionally found to act as a competitive inhibitor of α-chymotrypsin, a protease enzyme, with an IC_{50} of 188.8 μM.

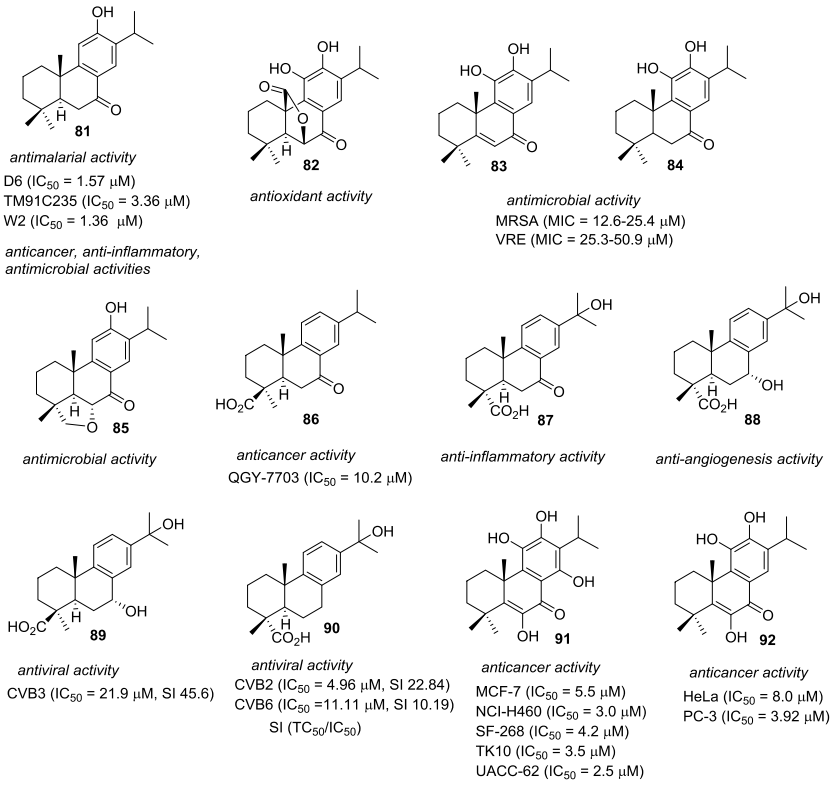


Figure 9 *Naturally occurring oxygenated aromatic abietanes with various bioactivities.*

Naturally occurring 13-propenyl-substituted aromatic abietanes are shown in Figure 10. Angustanoic acid E (**93**) demonstrated anti-inflammatory activity *in vitro* evaluated by measuring the inhibitory ratios of *β*-glucuronidase release in rat PMNs induced by PAF.[144] Jiadifenoic acid B (**94**) isolated from the roots of *Illicium jiadifengpi* B.N. Chang showed antiviral activity against Coxsackie virus B2, B3, B4, and B6.[22] Aquilarabietic acid H (**95**), exhibited antidepressant activity *in vitro* in a preliminary study measuring the inhibition of [^{3}H]-5-hydroxytryptamine and [^{3}H]- norepinephrine reuptake in the rat brain synaptosomes. At 10 μM, **95** inhibited [^{3}H]-NE reuptake by 73.8%. The authors hypothesized that the α-hydroxyl at position 7 might be responsible for the activity since all active compounds in this study possessed this structural moiety.[21]

Finally, Gao *et al.* recently reported the isolation of two new 13-propenyl dehydroabietanes from *C. bodinieri*,[124] namely bodinieric acids C (**96**) and F (**97**) of which bodinieric acid F showed moderate activity as spleen tyrosine kinase (SYK) inhibitors. The biological activity of antiochic acid (**98**) has not yet been explored.

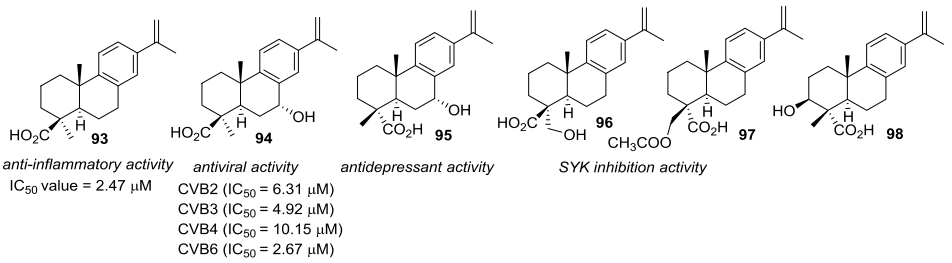
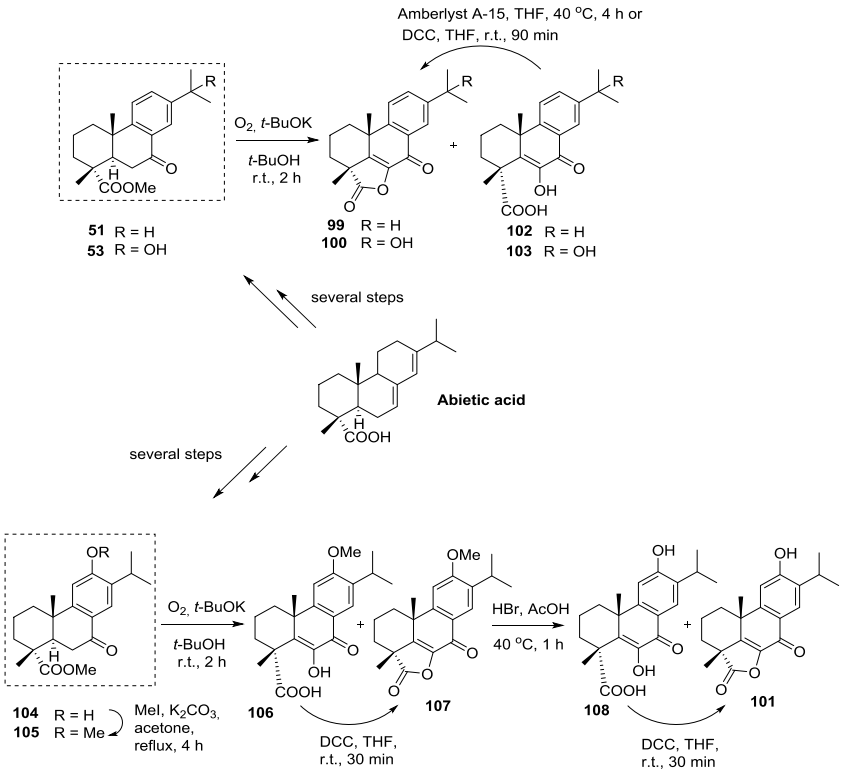


Figure 10 *Naturally occurring 13-propenyl-substituted aromatic abietanes.*

2.4.2 Semisyntheses of naturally occurring picealactones A, B and C and jiadifenoic acid C

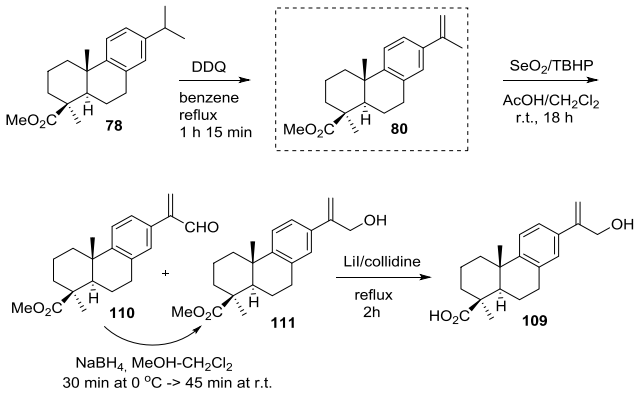
Alvarez-Manzaneda *et al.* developed semisynthetic routes for the naturally occuring picealactones A (**99**), B (**100**) and C (**101**) starting from abietic acid (Scheme 7).[96,98] These natural products exist in the heartwood of *Picea morrisonicola* [145] and bear a 5-dehydro-18,6-olide moiety common to the *ent*-kaurane stevionolide from *Stevia lucida* Lag, and the biological significance of this particular feature was unknown.[146] The semisynthesis of picealactones A and B proceeds via the 7-oxo-derivative **51** and 7-oxo-15-hydroxy derivative **53** prepared from abietic acid in several steps including chromium(VI) oxidation with PCC to obtain the 7-oxo functionality. Treatment of **51** with potassium *tert*-butoxide in *tert*-butanol under oxygen stream produced a 1:2 mixture of lactone **99** and disphenol **102**, which after chromatographic purification gave pure picealactone A (**99**). For the 15-hydroxy derivative **53**, the same treatment gave a 1:1 mixture of lactone **100** and disphenol **103**. Reaction with either Amberlite A-15 or DCC in THF produced picealactone B (**100**), as a single product, in 95-97% yield.

The semisynthesis of picealactone C occurs similarly via the 7-oxo derivative prepared with Na_2CrO_4. Methylation of **104** with iodomethane in the presence of potassium carbonate gave **105** in 95% yield which was treated with potassium *tert*-butoxide in *tert*-butanol, under an oxygen stream, giving a 1:1 mixture of disphenol **106** and lactone **107**. Treatment with DCC in THF produced the lactone **107**, as a single product, in 75% yield. An attemp to hydrolyze the methoxy group with hydrobromic acid in acetic acid gave a mixture of disphenol **108** and **101** which after treatment with DCC produced the desired picealactone C (**101**), in 71% yield.



Scheme 7 *Semisynthesis of naturally occurring picealactones A (**99**), B (**100**) and C (**101**) from abietic acid via a 7-oxo-dehydroabietate intermediate.*

Gonzalez *et al.* have developed a short semisynthesis route for the naturally occuring jiadifenoic acid C (**109**) (Scheme 8).[22,127] The starting material **78** was obtained by esterification from callitrisic acid (4-epidehydroabietic acid) isolated from Moroccan sandarac resin. The key reaction step of the synthesis route is the formation of the 13-propenyl moiety present in jiadifenoic acid C. It is obtained in the reaction of **78** with DDQ, in refluxing benzene, producing **80**, in 45% yield, as an unseparable mixture with the remaining starting material, which was recovered after the next step. Allylic oxidation of **80** with SeO_2 and *tert*-butyl hydroperoxide as a co-oxidant gave the allylic alcohol **111** together with the aldehyde **110**. To maximize the yield and to allow easier separation, the mixture was reacted with sodium borohydride in a MeOH-CH_2Cl_2 mixture giving **111** with an overall yield of 65%, in two steps. Finally, treatment of **111** with LiI in refluxing collidine gave jiadifenoic acid C (**109**), in 75% yield, after chromatographic purification.



Scheme 8 *Semisynthesis of jiadifenoic acid C (**109**).*

In summary, oxygenated and 13-propenyl aromatic abietanes possess a variety of biological activities and represent useful intermediates in the semisynthesis of other natural products, illustrated here with the semisyntheses of the picealactones and jiadifenoic C.

3 Aims of the study

The aims of the study were to design and synthesize new dehydroabietic acid derivatives as well as to study and develop new synthetic methods, with a focus on catalysis and sustainability, within diterpenoid chemistry. In addition, the goal was to screen the dehydroabietic acid derivatives prepared for their ability to prevent the growth of pancreatic cancer cells and inhibit nitric oxide production and identify the best compounds for further studies.

The more specific aims of the research were

- To study the benzylic oxidation of aromatic abietanes using sodium chlorite in combination with aqueous *tert*-butyl hydroperoxide to produce oxygenated derivatives and apply the method for the semisynthesis of naturally occurring picealactones A, B and C (I)

- To study bismuth(III) triflate based dehydration of tertiary alcohols and apply the method for the semisynthesis of natural products isolated from *Pinus massoniana* (II)

- To design and synthesize new dehydroabietic acid derivatives and to study their activities against pancreatic cancer and inflammation *in vitro* (III)

4 Results and discussion

4.1 Benzylic oxidation of dehydroabietanes with sodium chlorite and *tert*-butyl hydroperoxide

As presented in the literature review, toxic chromium(VI) reagents continue to be the number one option for the benzylic oxidation of aromatic abietanes. In our studies aiming towards finding alternatives for chromium, we identified sodium chlorite ($NaClO_2$) in combination with aqueous *tert*-butyl hydroperoxide (TBHP)[147] as a potential and environmentally sound oxidation method which has not been previously used for benzylic oxidations of aromatic abietanes. Sodium chlorite is a stable oxidizing agent mainly used for the bleaching and stripping of textiles, fibre, pulp and paper as well as for water disinfection.[148] In organic chemistry, oxidation systems based on sodium chlorite or hypochlorite and TBHP are considered as inexpensive and environmentally friendly water-based oxidation methods.[147,149]

In this regard, the optimal reaction conditions as well as scope and selectivity of this reaction were investigated. Initial studies were performed with methyl dehydroabietate **50** which is the most widely investigated compound for benzylic oxidations among aromatic abietanes. Compound **50** and most dehydroabietanes have two possible C-H positions to be oxidized, the cyclic secondary C-7 methylene and the exocyclic tertiary C-15. In addition, to highlight the utility of this method, a short synthesis route for the semisynthesis of naturally occurring picealactones A, B and C as well as to provide unprecented information about their bioactivity are presented.

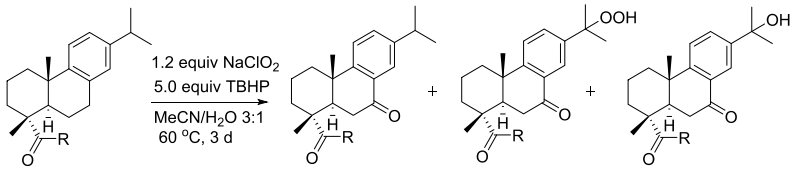
4.1.1 Optimization of the reaction conditions

In the beginning of the study, the reaction conditions were optimized, namely reaction time and the amount of oxidant. Detailed description of the optimization conditions is found from Table 1 in Publication I. When methyl dehydroabietate **50** was stirred for 1 day in a mixture of acetonitrile/water (3:1), in the presence of 1.2 equiv of $NaClO_2$ and 5.0 equiv of TBHP, three products formed, 7-oxo **51**, 7-oxo-15-peroxy **72** and 7-oxo-15-hydroxy **53** (Table 2). However, **51** had minor amounts of a side product, which was identified as the 7-peroxy compound **66**, a possible intermediate for the formation of the 7-oxo compound.[150,151] Increasing the reaction time to 3 days, gave **51**, **72** and **53** as pure compounds after chromatographic purification, in 39%, 31% and 7% yields, respectively (Table 2).

Increasing the amount of $NaClO_2$ (2.4 equiv) or reaction time (7 days) did not result in significant changes in the product ratios, only a small shift towards the formation of slightly higher amounts of **72** and **53** occured. Running the experiment with 1.2 equiv. of $NaClO_2$ or 5.0 equiv of TBHP as single oxidants could not exhaust the starting material revealing that they are both necessary for the oxidation of **50**. Moreover, catalytic amount of $NaClO_2$ (0.1 equiv) with TBHP (5.0 equiv) was also not enough to exhaust all the starting material. To conclude, the best reaction condition to obtain the three oxidation products required three

days as well as 1.2 equiv of $NaClO_2$ and 5.0 equiv of TBHP. These conditions were also applicable to other DAA derivatives, namely the primary, secondary and tertiary amides **112**, **113** and **114** producing a similar pattern of oxidation products (Table 2). Oxidation of **112** produced slightly less of the 7-oxo-15-peroxy compound than the other amides, suggesting that it might be less stable.

Table 2 *Benzylic oxidation of methyl dehydroabietate and primary, secondary and tertiary amides.*

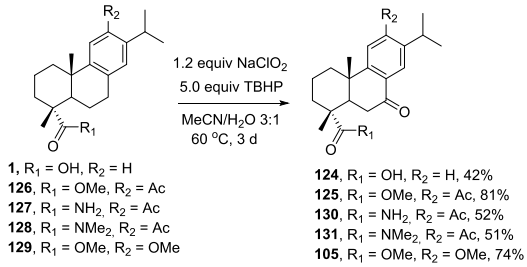


Substrate	R	Product	Yield (%)	Product	Yield (%)	Product	Yield (%)	Ratio of products
50	OMe	**51**	39	**72**	31	**53**	7	51:40:9
112	NH_2	**115**	30	**118**	9	**121**	13	58:17:25
113	$NHCH_2CO_2Me$	**116**	38	**119**	15	**122**	5	66:26:9
114	NMe_2	**117**	35	**120**	26	**123**	6	52:39:9

4.1.2 Selectivity of the reaction

A free radical mechanism is generally accepted for hydroperoxide- and chlorite-mediated allylic/benzylic oxidations where the determinant step is the formation of carbon radicals which can further react to form oxygenated products.[147,152] To confirm the reaction mechanism, an experiment with the optimized conditions was ran in the presence of the radical inhibitor butylated hydroxytoluene (BHT, 50 mol %). After 3 days, starting material was recovered along with small amounts (14%) of **51**, pointing out to the fact that the presence of BHT hampers the oxidation.

Direct oxidation of DAA was also possible with this method (Scheme 9). Surprisingly, only the 7-oxo product **124** formed. The yield remained relatively low (42%), however, this result is encouraging as the direct oxidation has been previously reported only by chromium-based methods. For 12-substituted derivatives, this method is regioselective producing only the 7-oxo products, in good yields, with various starting materials (Scheme 9). Especially high yield was obtained with **125** (81%). Both electron-withdrawing and -donating groups are well tolerated at position 12, as tested with acetyl and methoxy substituents, respectively. Preparation of the 12-substituted starting materials **126**-**129** is described in the supporting information of Publication I.



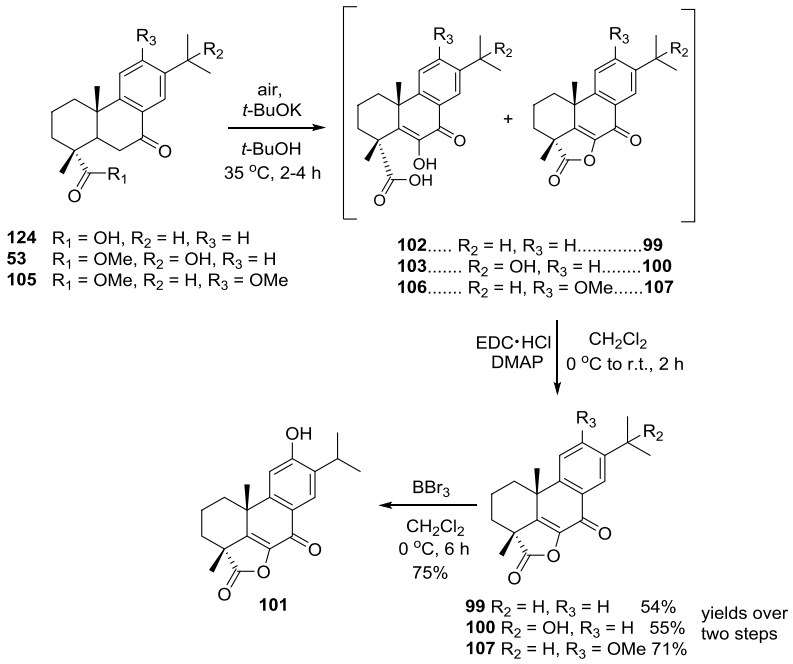
Scheme 9 *Regioselective oxidation of aromatic abietanes **1** and **126-129**.*

During our studies, the stability of the dehydroabietyl radicals at C7 and C15 of compounds **50**, **126** and **129** was calculated by *ab initio* computational studies to explain the observed regioselectivity. Indeed, the energy difference between the C7 and C15 radicals is much higher for compounds **126** (-7.917 kcal/mol) and **129** (-7.009 kcal/mol) than for compound **50** (-2.003 kcal/mol), indicating that 7-oxo derivatives form preferably in the case of 12-substituted derivatives. This study also showed that the free energy is lower for C7 radical compared to C15 supporting the observation that 7-oxo products form in bigger amounts regardless of the starting material. More details of the thermochemical calculations are found from Publication I and its supporting information.

4.1.3 Semisynthesis and biological activities of picealactones A, B and C

Previous studies[96,153] report the semisynthesis of picealactones A, B and C starting from abietic acid in multiple steps as presented in the literature review. Also, another recent study[154] reports the synthesis of picealactone A from DAA in 3 steps via chromium(VI) oxidation. With our synthetic route, picealactones A and B were prepared in 3 or 4 steps, respectively, starting from a commercially available compound. Picealactone C required additional steps due to the need to introduce a 12-hydroxy group.

The oxidized derivatives **124**, **53** and **105**, prepared by $NaClO_2$/TBHP oxidation, were further oxidized using a modified procedure by Korovin *et al.*[155] with potassium *tert*-butoxide in *tert*-butanol while air was bubbled through the reaction mixture, to produce a mixture of the disphenol **102**, **103** or **106** and lactone **99**, **100** or **107** (Scheme 10). Treatment of the mixtures with EDC hydrochloride in the presence DMAP gave the desired picealactones A (**99**) and B (**100**), in 54% and 55% yields (over two steps), respectively, as well as the lactone **107** in 71% yield (over two steps). Finally, *O*-demethylation of **107** with BBr_3 gave the picealactone C (**101**), in 75% yield.



Scheme 10 *Semisynthesis of picealactones A (**99**), B (**100**) and C (**101**).*

In the antiproliferative screening, picealactones A and C inhibited the proliferation of prostate (PC-3) and breast (T47D) cancer cell lines with IC_{50} values close to 10 μM (Table 3). Picealactone B was inactive indicating that the free hydroxyl group at position 15 has somewhat negative effect on the proliferation activity, whereas in position 12 it does not seem to affect much of the activity compared to picealactone A. Thus, picealactones A and C can be regarded as good starting materials for further optimization in pursuit of new agents to target breast cancer.

Table 3 *IC_{50} values (μM) for picealactones A-C in PC-3 and T47-D cells.*

Compound	PC-3	T47D
Picealactone A (**99**)	59.9	10.3
Picealactone B (**100**)	>100	23.6
Picealactone C (**101**)	32.6	14.5

In conclusion, sodium chlorite in combination with *tert*-butyl hydroperoxide provides a convenient, inexpensive and environmentally sound option for chromium(VI) reagents for the benzylic oxidation of aromatic abietanes. Especially for 12-substituted compounds, good yields and very pure products are obtained. Moreover, in one step we obtain three different oxidation products, potential compounds to be included into chemical libraries to screen against different drug targets. Furthermore, this method is applicable for the

semisynthesis of naturally occurring diterpenoids, demonstrated here as the picealactones A, B and C, otherwise inaccessible for bioactivity studies.

4.2 Bismuth(III) triflate-mediated dehydration of tertiary alcohols

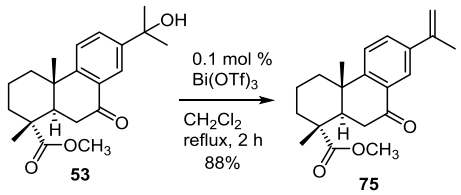
Dehydration of alcohols into the corresponding alkenes is an important reaction in organic chemistry. Our interest towards dehydration of tertiary alcohols started as we considered suitable methods to build the 13-propenyl functionality onto aromatic abietanes. This functionality exists in several known natural products as summarized in the literature review. We identified bismuth(III) triflate as a suitable reagent to perform the dehydration of tertiary alcohols into the corresponding alkenes with very low catalyst loadings.

Bismuth(III) salts are versatile reagents for a variety of organic reactions,[156–160] including syntheses of pharmaceutically interesting compounds and natural products.[161–165] In addition, they are relatively non-toxic and easy to handle, and therefore bismuth(III)-based salts are considered as environmentally sound reagents. $Bi(OTf)_3 \cdot xH_2O$ is one of the most commonly used bismuth(III) salt, because it is commercially available, inexpensive and can promote both Lewis and Brønsted acid catalysis.[160,166]

Herein, the studies about bismuth(III) triflate as an alcohol-dehydrating agent are presented. Optimization of the reaction conditions revealed that the reaction solvent and its polarity have crucial roles in the reaction outcome. In apolar solvents, alkenes are obtained, in high yields, whereas in polar solvents dimerization occurs. Moreover, we show that this method is applicable to compounds from different chemical classes. Finally, the utility of the novel method for the semisynthesis of known natural products existing in *Pinus massoniana* is demonstrated.

4.2.1 Optimization of the reaction conditions and mechanism studies

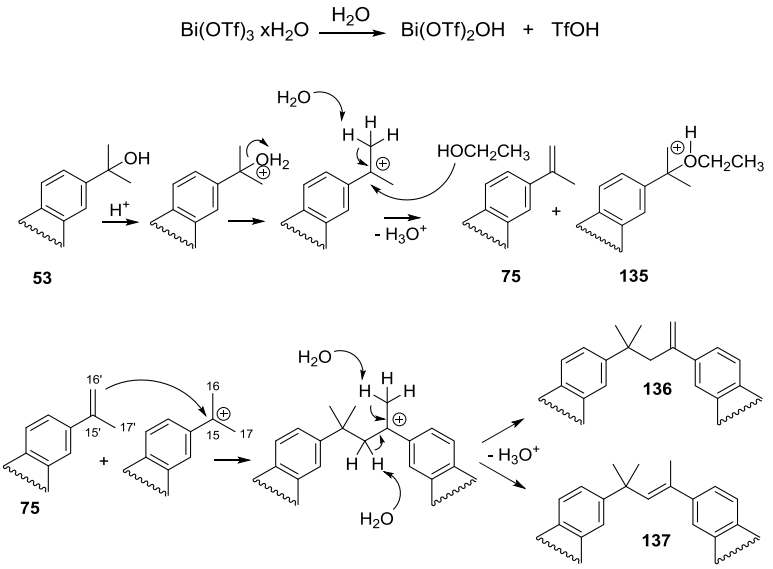
In the beginning of the study, the optimal reaction conditions for the dehydration of tertiary alcohols with bismuth(III) triflate using **53** as a starting material were investigated (Scheme 11). Herein a summary of the most important findings is presented. More details can be found from Table 1 in Publication II. Chloroform was first used as the reaction solvent and the best result was obtained with 0.1 mol % of $Bi(OTf)_3 \cdot xH_2O$, in 24 hours, at reflux with gain in yield (85%) from the initial 5 mol % and 2-hour reaction time (63%). Formation of a side product **135** (Scheme 12) occurred when ethanol, which is used to stabilize chloroform, reacted with the formed carbocation intermediate. The use of larger amounts of $Bi(OTf)_3 \cdot xH_2O$ produced more side product whereas in dichloromethane, no side product formed, and the desired product was obtained under the same conditions, in 2 hours, in 88% yield.



Scheme 11 *The optimized reaction conditions for the dehydration of **53**.*

The role of ethanol in the reaction was further investigated, leading to a better understanding of the reaction mechanism. In dry ethanol, increasing amounts of **135** formed when the amount of catalyst was raised from 1-20 mol %. With non-dry ethanol, a major change in the reactivity occurred. Compound **135** did not form, but instead, a mixture of the two dimeric compounds **136** and **137** was obtained (Scheme 12).

Moreover, solvent polarity appeared to be an important parameter towards the reaction outcome. Other solvents such as 1,4-dioxane, THF, acetonitrile and nitromethane were also tested. In the absence of water **136** and **137** did not form and the use of non-polar solvents such as 1,4-dioxane and dichloromethane as well as the relatively non-polar THF favored the formation of the alkene derivative **75**. In polar, non-dry solvents such as acetonitrile or nitromethane, in a similar fashion to ethanol, compounds **136** and **137** became the major reaction products. Ideal conditions to prepare **136** were with 1 mol % of $Bi(OTf)_3 \cdot xH_2O$ in refluxing nitromethane, which gave pure **136**, in 71% yield.



Scheme 12 *Proposed reaction mechanism.*

Based on the findings of this study, bismuth(III) triflate behaves as a Brønsted instead of Lewis acid meaning that triflic acid, produced *in situ* from $Bi(OTf)_3 \cdot xH_2O$, protonates the

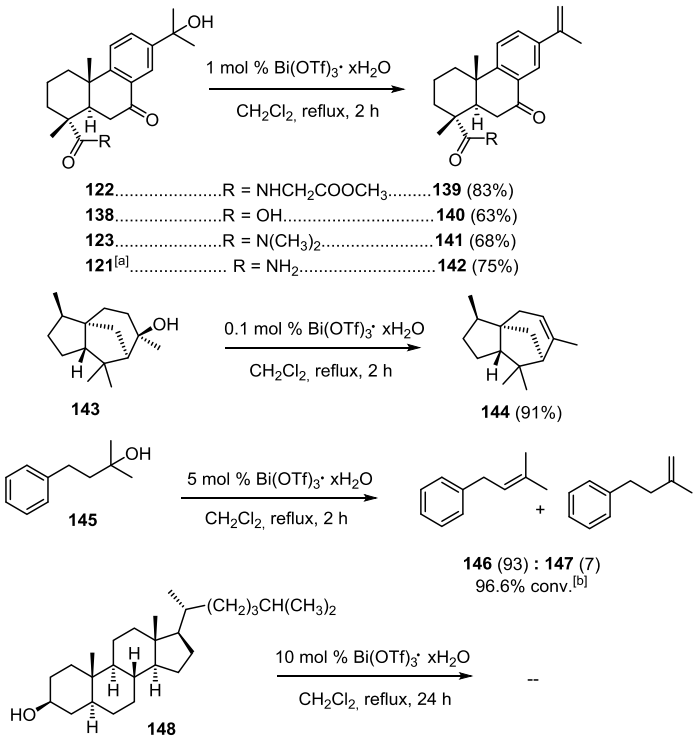
hydroxyl group which leaves as water, producing the stable tertiary carbocation (Scheme 12). Notably, evidence for this behavior was provided by the fact that in the presence of a proton scavenger 2,6-di-*t*-butylpyridine (10 mol %)[167] the reaction did not progress, and on the other hand, with triflic acid (10 mol %), full conversion of **53** into compounds **75**, **136** and **137** occurred. Compound **135** forms in chloroform only due to the presence of ethanol as a chloroform stabilizer, which attacks as a nucleophile to the formed carbocation. Dimers **136** and **137** form after electrophilic addition of the carbocation to the double bond of **75** followed by the removal of one of the protons by water (Scheme 12). In non-dry solvents, water originates most likely from hygroscopic bismuth(III) triflate. The formation of **136** and **137** is in line with previous reports describing acid-catalyzed dimerization of styrene[168] as well as dimerization via proton transfer to carbonyl compounds.[169] The "super acid" triflimide has also been reported to promote the hydroalkenylation of vinylarenes.[170] In all cases, the reaction mechanism goes via formation of the benzylic cation which reacts with the vinylarenes through nucleophilic addition. Finally, deprotonation affords the dimeric products.

Promotion of the direct homodimerization of **75** by bismuth(III) triflate was also tested as a previous study reported on the heterodimerization of vinylarenes where indium triflate had been used as a Lewis acid catalyst.[171] However, regardless of several attemps in different solvent systems (1,4-dioxane, 3:1 THF/cyclohexane, nitromethane),[170] only small amounts of the mixture of **136** and **137** formed in nitromethane with 5 mol %, and in 1,4-dioxane, with 10 mol % of the catalyst.

4.2.2 Scope of the reaction

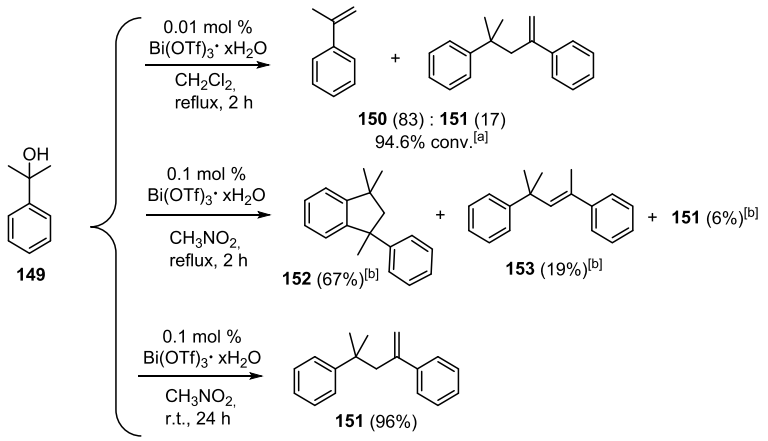
Screening of different metal salts confirmed that bismuth(III) triflate is the most efficient for this transformation. Triflates of copper, scandium, ytterbium, lanthanum and indium were tested as well as bismuth(III) chloride and bromide. However, in all cases 5-20 mol % were necessary to exhaust all the starting material, and for ytterbium and lanthanum(III) triflate even with 20 mol %, approximately 50% of the starting material remained unmodified. More details of the screening are provided in Table 2 of Publication II.

Other diterpenic benzylic tertiary alcohols, differing from the functional group at position 18, were also successfully transformed into the corresponding alkene derivatives with $Bi(OTf)_3 \cdot xH_2O$ (Scheme 13). Slightly higher amounts of the catalyst were necessary to exhaust all the starting material probably due to presence of atoms which coordinate with bismuth(III) triflate affecting its catalytic power. Moreover, dehydration of the sesquiterpene cedrol (**143**) with 0.1 mol % of $Bi(OTf)_3 \cdot xH_2O$ gave cedrene (**144**) in 91% yield. Tertiary alcohol **145** gave alkenes **146** and **147** in a ratio of 93:7, with 96.6% conversion, however, with a higher amount of catalyst, reflecting the lower reactivity of non-benzylic alcohols towards this method. In line with this finding, the reaction was unsuccessful for dehydration of secondary alcohol 5α-cholestan-3β-ol (**148**).



Scheme 13 *Scope of the reaction. [a]5 mol % Bi(OTf)₃·xH₂O was used. [b]Determined by gas chromatography-mass spectrometry.*

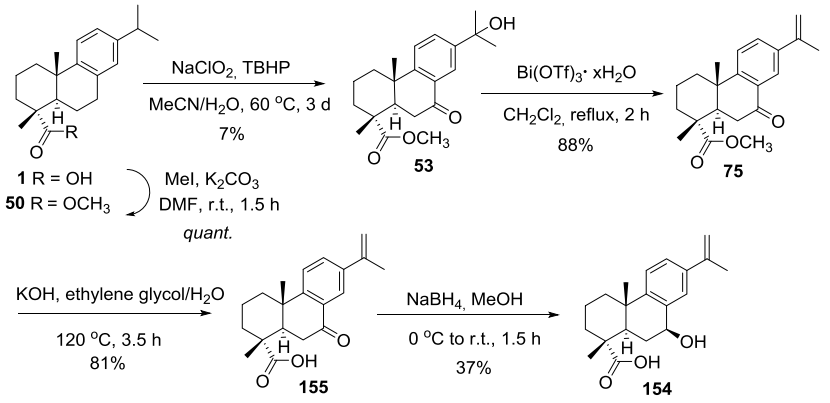
Alcohol **149**, on the other hand, gave the alkene **150** and the dimer **151**, with 94.6% conversion, in an 83:17 ratio, with only 0.01 mol % of the catalyst (Scheme 14). When the reaction solvent was changed to nitromethane the indane derivative **152** formed with 0.1 mol % catalyst, in 67% yield, after 2 hours, along with **153** and **151** as minor products. This result is consistent with a previous study that reports the preparation of **152** from **149** in the presence of equimolar amounts of $BiBr_3$ in chloroform, after cyclization of the linear dimer **151**, at high temperature.[172] Interestingly, at room temperature using $Bi(OTf)_3 \cdot xH_2O$ as catalyst, in nitromethane, **151** was obtained from **149**, as the single reaction product, in 96% yield. Of note, cyclization to the indane derivatives was never observed with the larger molecules such as the diterpenoid **53** most likely because of steric effects.



Scheme 14 *Treatment of **149** with 0.01 mol % or 0.1 mol % of $Bi(OTf)_3 \cdot xH_2O$ in dichloromethane or in nitromethane produces alkene, dimers or indane products. [a]Determined by gas chromatography-mass spectrometry. [b] Calculated from the ^1H NMR spectrum.*

4.2.3 Semisynthesis of natural products from *Pinus massoniana*

Finally, the previously described new method was used for the semisynthesis of naturally occurring 13-propenyl-7-hydroxyabieta-8,11,13-trien-18-oic acid (**154**) existing in *Pinus massoniana* (Scheme 15). The semisynthesis started from compound **1**, which was first converted into the methyl ester **50**, followed by the $NaClO_2$/TBHP-mediated oxidation presented in the Publication I to give **53**. The alkene **75** was obtained by bismuth(III) triflate-mediated dehydration. After the hydrolysis of the methyl ester of **75** with potassium hydroxide in refluxing ethylene glycol, the carbonyl group at position 7 was reduced with sodium borohydride to give the desired product **154**, in 37% yield, in 5 steps. The α-orientation of H-7 was assigned by the presence of a NOESY-correlation of H-5/H-7 and the broad triplet at 4.75 (t, J = 8.8 Hz) consistent with a previous study.[173]



Scheme 15 *Semisynthesis of 13-propenyl-7-hydroxyabieta-8,11,13-trien-18-oic acid (**154**).*

To conclude, it was shown that bismuth(III) triflate provides an excellent option for the dehydration of tertiary alcohols and is applicable to compounds from different chemical classes bearing various functional groups. In polar solvents, bismuth(III) triflate selectively catalyzes the dimerization of the alcohols instead, with the formation of new C-C bonds, in yields up to 96%.

4.3 Dehydroabietic acid derivatives targeting pancreatic cancer and cancer-related inflammation

Because targeting pancreatic cancer is extremely challenging, development of multifunctional compounds capable of reaching several relevant drug targets was considered. These drug targets may modulate entire regulatory networks or multiple pathways, being both preventive and therapeutic, and could serve as an effective treatment for this devastating disease.

For instance, SOs are considered as multifunctional compounds. According to several preclinical animal model studies, both natural and semisynthetic triterpenoids act at various stages of tumor development including inhibiting initiation and promotion of carcinogenesis, inducing tumor cell differentiation and apoptosis, and suppressing tumor angiogenesis.[11,174–178] Two triterpenoid oleanolic acid derivatives, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and CDDO-Me, progressed into phase I clinical trials for the treatment of leukemia as well as solid tumors and lymphoid malignancies.[43,179,180]

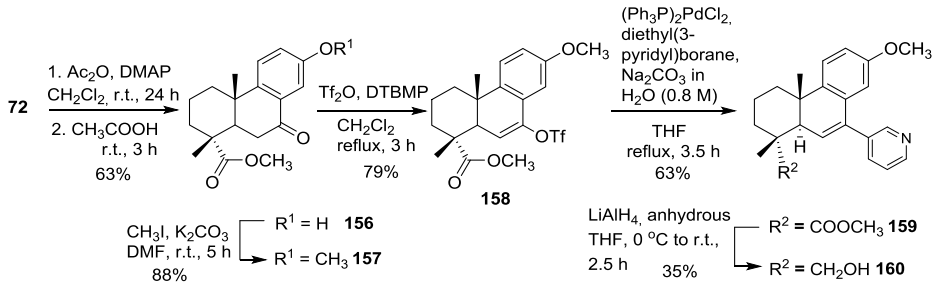
Inspired by these studies, we became interested in developing and synthesizing DAA derivatives against pancreatic cancer, since DAA and its semisynthetic derivatives have shown anticancer activity against several cancer types as well as anti-inflammatory activity. In addition, DAA provides a good molecular scaffold for further chemical modifications, as reviewed in the literature part. Moreover, so far very little is known about the potential of DAA and its derivatives to target pancreatic cancer.

Sets of novel DAA derivatives were synthesized and screened for their antiproliferative activity in human and mouse pancreatic cancer cell lines as well as their ability to block the formation of nitric oxide. Based on the preliminary screening, we selected the most potent compounds and tested their ability to induce cell differentiation and carried out target deconvolution studies to propose a possible mode of action. In addition, western blot studies were conducted to test whether the compounds affect the expressions of cell cycle proteins cyclin D1 and p27.

4.3.1 Design, synthesis and preliminary screening of the compounds

First, some of the previously synthesized oxidation products from Publication I as well as compounds **156** and **157** (Scheme 16) were tested. Removal of the C13 isopropyl side chain was accomplished in two steps by treating **72** first with acetic anhydride (Ac_2O) in the presence of DMAP and then refluxing the acetylated intermediate in acetic acid to give the

phenol **156**, in 63% yield (over two steps), which was subsequently converted into **157**, in 88% yield.



Scheme 16 *Removal of the C13 isopropyl group and synthesis of the pyridyl derivatives **159** and **160**.*

In the preliminary screening, compounds were tested for their ability to inhibit the proliferation of human Aspc-1 and murine PanAsc 2159 pancreatic cancer cells using the MTT assay (Table 4). Moreover, their ability to block the formation of NO, an important mediator of inflammation in the tumor microenvironment, was examined using mouse macrophage-like cells (RAW 264.7). From the first set of compounds, only the oxidation products **51** and **125**, both bearing a carbonyl group at position 7, showed modest activity against the pancreatic cancer cell lines, whereas **53** was active only against Aspc-1 cells with an IC_{50} of 19.3 μM. DAA (**1**) which acted as the control was inactive in all assays, even when tested at the high concentration of 60 μM.

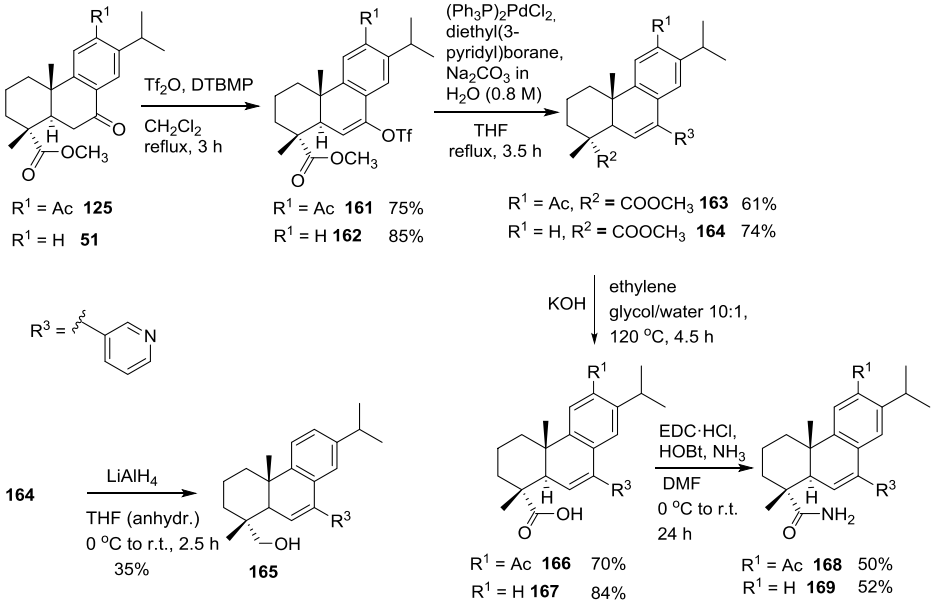
Table 4 *Anti-proliferative and anti-inflammatory activity screening of DAA and its oxidation products. The values are the mean ± SD of at least three independent experiments.*

Compound[a]	IC_{50} (μM)		
	PanAsc 2159[b]	Aspc-1[b]	NO[c]
51	28.3±3.1	31.0±0.4	N.A.[d]
53	N.A.	19.3±1.8	N.A.
125	27.6±5.0	22.5±3.6	N.A.

[a]Compounds **1**, **156**, **157**, **126** and **72** were inactive against all parameters measured at the highest concentration tested (60 μM) [b]IC_{50} values were determined by MTT assay after 72 hours of treatment. [c]IC_{50} values were determined using RAW 246.7 mouse macrophage-like cells after treatment with the compound for 20 minutes and stimulation with INFγ (10 ng/mL) for 24 hours. [d] N.A. = not active at 60 μM.

It has been estimated that fifty-nine percent of small-molecule drugs contain nitrogen heterocycles, of which pyridine, piperidine, and piperazine represent the most common ones.[181] Pyridyl groups tend to increase metabolic stability[182] and have shown to improve the drug-like properties of natural products.[183] In addition, according to several studies pyridyl groups have improved anticancer properties of several semisynthetic terpenoids.[182] The main hypothesis was that coupling of a pyridyl group into position 7 of DAA could

improve its bioactivity. Pyridyl group was inserted by Suzuki cross coupling into compounds **158**, **161**, **162** prepared from the corresponding 7-oxo compounds with trifluoromethanesulfonic anhydride in the presence of di-*tert*-butylmethylpyridine (DTBMP). Coupling of the heterocycle was made with bis(triphenylphosphine)palladium(II) dichloride and diethyl(3-pyridyl)borane, in the presence of a 0.8 M aqueous solution of sodium carbonate (Scheme 16, Scheme 17).[28] Compounds **159**, **163** and **164** were obtained in 74%, 61% and 63% yields, respectively, after chromatographic purification. Position 18 was further functionalized to test its effect on the activities. Reduction of the ring A ester in **159** and **164** with lithium aluminium hydride gave alcohols **160** and **165**, whereas hydrolysis with potassium hydroxide in a mixture of ethylene glycol and water (10:1) gave acids **166** and **167** that were further converted into amides **168** and **169** through carbodiimide coupling.



Scheme 17 *Synthesis of the pyridyl derivatives **165-169**.*

In the set of pyridyl derivatives, ester **164** and alcohol **165** were most active against both pancreatic cancer cell lines and inhibited the production of NO with IC_{50} values ranging from of 20 to 23.5 μM (Table 5). The amide **169** was slightly less active, with IC_{50} values varying from 30.3 to 34.0 μM. In the same concentration range (32.9 to 38.5 μM) was also the ester **159** with the modified C13 side chain. Of the 12-acetyl derivatives only **163** was active against murine PanAsc 2159 cells with an IC_{50} value of 23.9 μM and could in addition block NO production within the same concentration range. Other 12-acetyl derivatives (**166**, **168**) were inactive. The presence of a carboxyl group in ring A (**166** and **167**) had a negative impact on the anti-proliferative activity of these compounds, however, the ability to inhibit NO production was retained in **167**. In most cases, insertion of the pyridyl group improved

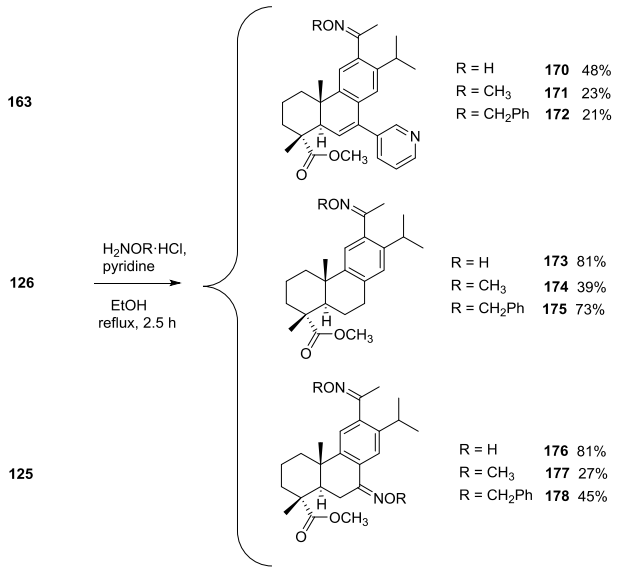
the activity. Nevertheless, as the potencies still remained in the high micromolar range, an additional set of compounds were synthesized.

Table 5 *Anti-proliferative and anti-inflammatory activity screening of the pyridyl derivatives of DAA. The values are the mean ± SD of at least three independent experiments.*

Compound[a]	IC_{50} (μM)		
	PanAsc 2159[b]	Aspc-1[b]	NO[c]
159	38.5±7.1	35.9±3.0	32.9±0.6
163	23.9±4.4	N.A.	29.9±1.3
164	22.9±1.9	20.0±2.4	23.0±0.3
165	22.0±2.3	20.3±2.7	23.5±0.2
167	N.A.	N.A.	27.8±0.7
169	34.0±3.5	30.3±3.6	31.2±2.0

[a]Compounds **160**, **166** and **168** were inactive against all parameters measured at the highest concentration tested (60 μM). [b]IC_{50} values were determined by MTT assay after 72 hours of treatment. [c]IC_{50} values were determined using RAW 246.7 mouse macrophage-like cells after treatment with the compound for 20 minutes and stimulation with INFγ (10 ng/mL) for 24 hours. [d] N.A. = not active at 60 μM.

Several studies report that oxime and *O*-substituted oxime derivatives possess potential anti-inflammatory and anticancer properties, including activities against multi-drug resistant cancer cell lines.[184–190] In addition, it was interesting to see how changing the chemical properties of position 12 substituent from a mere hydrogen bond accepting group into an oxime, which has both hydrogen bond donating and accepting atoms, would affect the activitities. Thus, we decided to synthesize a group of oxime derivatives using **163**, **125** and **126** as starting materials. The carbonyl group at position 12 (**163**, **126**) or positions 7 and 12 (**125**) was modified into either a simple oxime(s) or an *O*-substituted oxime(s) according to Scheme 18. Reaction of **163**, **126** or **125** with hydroxylamine hydrochloride or the corresponding *O*-alkyl hydroxylamine in the presence of pyridine gave the oximes **170**-**178**.



Scheme 18 *Synthesis of dehydroabietic oxime derivatives.*

Introduction of an oxime into position 12 clearly increased activity against both pancreatic cancer cell lines as well as the inhibition of NO production, with and without the pyridyl group at position 7 (Table 6). Compound **173** displayed the most potent anti-inflammatory effects, with an IC_{50} value of 1.1 μM. Compound **170** was the second best with an IC_{50} value of 6.8 μM. In Aspc-1 cells, the IC_{50} values were reduced to low micromolar range in **170** (8.6 μM) and **173** (8.9 μM). The bulkier oximes **174**, **175**, **177** and **178** were inactive. However, in the presence of the 7-pyridyl group, **171** and **172** showed improved activity in all assays with IC_{50} values ranging from 8.6 to 15.9 μM.

Table 6 *Anti-proliferative and anti-inflammatory activity screening of the dehydroabietic oxime derivatives. Values are the mean ± SD of at least three independent experiments.*

Compound[a]	IC_{50} (μM)		
	PanAsc 2159[b]	Aspc-1[b]	NO[c]
170	10.6±3.0	8.6±1.1	6.8±1.6
171	15.9±4.6	14.0±1.1	12.2±4.6
172	11.2±3.5	8.6±2.6	11.6±1.6
173	14.8±1.4	8.9±1.2	1.1±0.6
176	16.4±1.1	13.1±0.6	10.8±2.8

[a]Compounds **174**, **175**, **177** and **178** were inactive against all parameters measured at the highest concentration tested (60 μM). [b]IC_{50} values were determined by MTT assay after 72 hours of treatment. [c]IC_{50} values were determined using RAW 246.7 mouse macrophage-like cells after treatment with the compound for 20 minutes and stimulation with INFγ (10 ng/mL) for 24 hours. [d] N.A. = not active at 60 μM.

4.3.2 Western blotting, cell cycle analysis, differentiation and kinase profiling studies

As the compounds inhibited the growth of pancreatic cancer cells, we were interested to see whether this was due to the effects on the regulation of the cell cycle. Thus, we conducted western blot and cell cycle analyses with five oxime derivatives, **170-173** and **176**, selected based on the *in vitro* screening results. PanAsc 2159 and Aspc-1 cells were treated for 48 hours with the compounds and the protein expression of p27 and cyclin D1 was investigated. Deregulation of cyclin dependant kinases (CDKs) by mutations or abnormally high expressions of cyclins, such as cyclin D1, is common in many cancers. High levels of cyclin D1 lead to its binding to CDK4 and CDK6 and cell progression from phase G1 into S phase where copying of the DNA will begin.[191–193] p27 on the contrary acts as a cyclin-dependent kinase inhibitor regulating the activity of CDKs. Low levels of p27 are generally associated with several cancers, including pancreatic cancer.[194]

The study showed that in PanAsc 2159 cells (Figure 11B) compounds **170-173** and **176** downregulated the expression of cyclin D1 in a dose-dependent manner and compounds **170**, **173** and **176** upregulated p27 expression. Compound **1** had no effect on the expression of either of the proteins, even at 20 μM. Aspc-1 cells (Figure 11A) did not express cyclin D1 but p27 was upregulated in a dose-dependent manner for all tested compounds apart from **1** where the effect was the opposite at 20 μM. The cell cycle analysis on Aspc-1 cells showed that with compounds **170-173** and **176**, especially at 10 μM concentration, the majority of the cells are in the G1 phase (Figure 11E, Figure 11F). The histogram (Figure 11C) shows the results for compound **176** *vs.* control. In PanAsc 2159 cells (Figure 11D) a similar trend is observed, although not so evident. These findings together with the anti-proliferative data support the fact that the compounds most likely induce cell cycle arrest at the G1 phase.

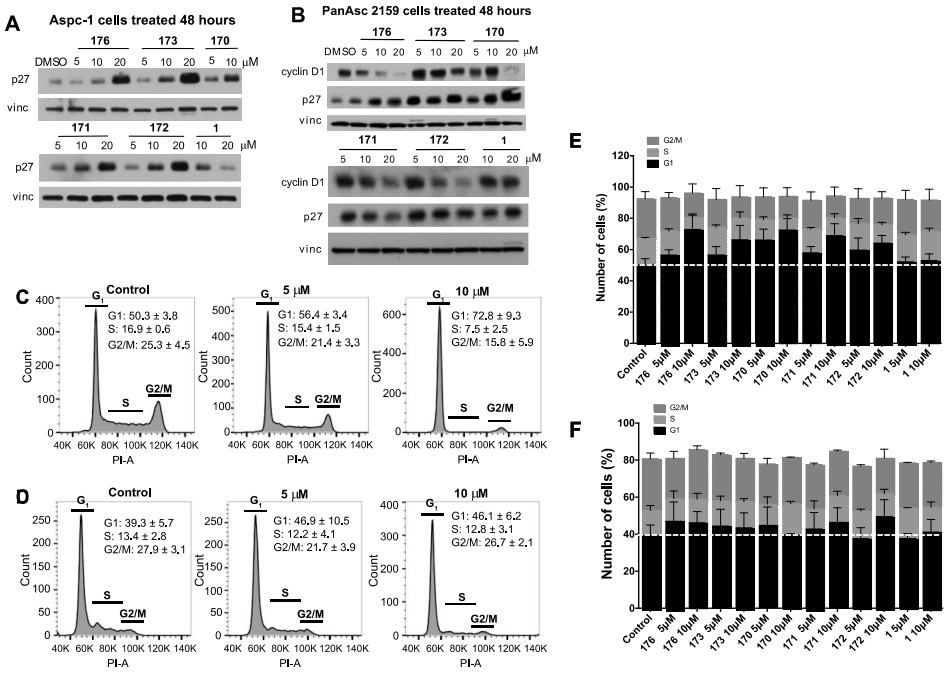


Figure 11 *Effects of on the expression of cell cycle proteins p27 in Aspc-1 cells (**A**) and p27 and cyclin D1 in PanAsc 2159 cells (**B**). Blots have been grouped from different parts of the same gels. Full-length blots and multiple exposures are found in the Supplementary Information. For compound **170**, concentration 20 μM was cropped out (from Aspc-1 treatment) as there was not enough protein to get a comparable result (most cells died at 20 μM). Vinc: vinculin (loading control). Cell cycle analysis of pancreatic cancer cells treated with compounds **170-173**, **176** and **1**. Aspc-1 (C, E) and PanAsc 2149 (D, F) cells were treated with at 5 and 10 μM of the compounds for 48 hours. The histogram of **176** is illustrated (C, D). Results for the different stages of cell cycle are indicated for 3-4 independent experiments (mean ± SEM).*

Regulating cell differentiation is highly desirable for cancer prevention and treatment.[195] The possibility to find compounds that would push malignant cells to overcome the block of differentiation and steer them into apoptosis instead of using cytotoxic agents could provide better treatment outcomes and less toxicity. We evaluated the ability of the compounds to induce monocyte differentiation in U937 human leukemia cells using CD11b, a cell surface antigen, as a differentiation biomarker. This marker is weakly expressed on leukemia U937 cells but can be induced with various established differentiation agents such as retinoids or rexinoids.

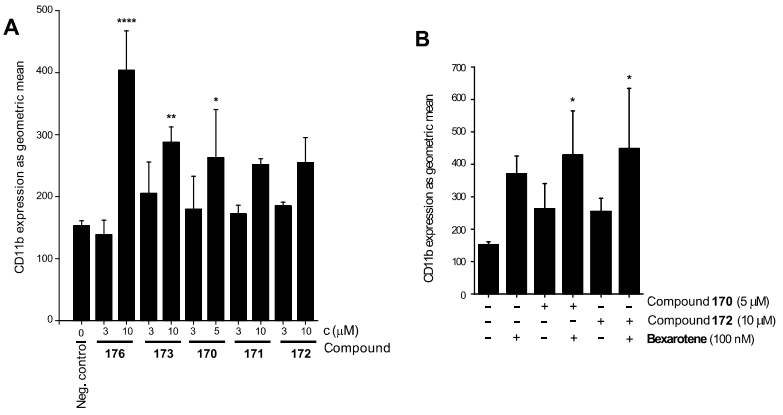


Figure 12 *Ability of compounds **170-173** and **176** to induce cancer cell differentiation in U937 cells alone (A) and ability of compounds **170** and **172** to induce cancer cell differentiation in U937 cells in combination with bexarotene (B). *P < 0.05, ** P < 0.001, *** P < 0.0001 vs. vehicle (DMSO) alone.*

After treating the cells for 4 days with the compounds, the expression of CD11b was measured by flow cytometry. As shown in Figure 12A induction of CD11b increased with all compounds, in a dose-dependent manner, when compared with the control. Compound **176** was the most efficient in inducing CD11b expression, particularly evident at 10 μM. Compounds **170** and **172** were also studied in combination with bexarotene (100 nM, Figure 12B), the only FDA-approved rexinoid for the treatment of cutaneous T-cell lymphoma, to look for possible synergistic effects.[196,197] Unfortunately, no synergistic effects occurred in combination with bexarotene.

Finally, two oxime derivatives, **170** and **173** were screened against a panel of 140 kinases at 10 μM concentration at the International Centre for Kinase Profiling (ICKP), in Dundee, UK (Publication III, Table S1, Supporting information). Neither of the compounds appeared as an outstanding kinase inhibitor and the maximum level of enzyme inhibition remained at 30-40%. However, compound **173** showed activity against only one of kinases, namely the p90 ribosomal S6 kinase 2 (RSK2), an AGC kinase of the RSK family. Although inhibition remained modest (34%), it is still encouraging as no concomitant inhibition of RSK1, one of the four known isoforms, appeared. **170** exhibited, in addition to RSK2, comparable activity against five other kinases (Table 7).[198,199]

Table 7 *Kinase profiling studies. Results of selected kinases are presened here. Values present the remaining enzyme activity ±SD after treatment with the compounds **173** and **170**.*

Entry	Kinase	% Activity ± SD (10 μM)	
		Compound **173**	Compound **170**
1	RSK1	84 ± 16	98 ± 5
2	RSK2	66 ± 15	65 ± 9
3	PKBβ	90 ± 4	60 ± 2
4	IKKβ	85 ± 10	70 ± 17
5	MST2	86 ± 5	73 ± 11
6	Src	90 ± 2	72 ± 6
7	BTK	87 ± 1	71 ± 0

RSKs (1-4) are a group of Ser/Thr kinases that act as downstream mediators of the Ras-ERK signal transduction pathway. They phosphorylate many substrates, including p27, involved in cell differentiation, survival, growth and proliferation.[198–200] High levels of RSK1 and/or RSK2 are expressed in several cancers, promoting tumor growth and survival. Thus, they are seen as promising cancer drug targets. Currently, none of the developed RSK inhibitors reported in the literature is selective towards one isoform.[199–202]

In this regard, the selective effect of compound **173** on RSK2 activity seems very promising. In addition, the loss of selectivity in **170** provides important preliminary data to small chemical modifications around the diterpenoid core that could impact this parameter. However, it is noteworthy that the SD for the inhibition of RSK2 for **173** was ±15 so repeating this study should be considered. Possible concomitant inhibition of RSK2 and PKBβ seen with **170** would also need to be tackled due to the specific role of the latter in insulin signaling. Nonetheless, dehydroabietic oximes can be regarded as a new chemical class with potential to develop isoform-selective RSK inhibitors.[198]

Structural modifications to the dehydroabietane core showed that oxime at position 12 was essential for the anticancer and anti-inflammatory activities. Furthermore, pyridyl and oxime at position 7 also somewhat increased activity. A methyl ester was the best tolerated group for position 18. With this study, we showed that dehydroabietic acid provides a good scaffold for chemical modifications in search of new multifunctional agents for cancer prevention and treatment. Dehydroabietic oximes appeared as the most promising anticancer agents with several desirable properties being anti-proliferative, anti-inflammatory and differentiation-inducing.

5 Summary and conclusions

Natural product research has over the years resulted in the discovery of many important drugs to treat various diseases. Many natural products, such as abietane-type diterpenoids, serve as potential scaffolds to develop new agents for the treatment and prevention of cancer. The need for new treatment strategies for pancreatic cancer is evident as the death rates alarmingly increase suggesting it to become the second most common cancer by 2030. Chronic inflammation, one of the hallmarks of cancer, increases the risk of cancer pathogenesis and serves as one of the targeting options.

Naturally occurring oxygenated aromatic abietanes possess not only interesting biological activities for the development of new drug candidates but also provide good starting materials for the semisynthesis of novel derivatives aiming towards improving bioactivity. In this regard, we identified sodium chlorite in combination with aqueous *tert*-butyl hydroperoxide as a useful method to replace the most widely used chromium(IV) reagents. These reagents oxidize 12-substituted aromatic abietanes regioselectively producing the 7-oxo derivatives with good yields. Preparation of 7-oxo, 7-oxo-15-hydroperoxy and 7-oxo-15-hydroxy derivatives of various aromatic abietanes is also possible with this method.

Moreover, we demonstrated that this method can be applied for the short semisyntheses of the naturally occurring picealactones A, B and C. A small antiproliferative screening revealed that picealactones A and C had micromolar activity against breast cancer cells making them potential candidates for further optimization. Consequently, our study introduced a new method to replace the environmentally hazardous chromium(IV) reagents, still widely used for the benzylic oxidations of aromatic abietanes. It is noteworthy that the method is most useful for the 12-substituted derivatives where only one oxidation product is obtained unless the aim is to oxide the both benzylic positions and produce several oxidation products for biological screenings.

In the pursuit of new methods to produce 13-propenyl-substituted aromatic abietanes, a moiety existing in some of scarcely available aromatic abietanes, bismuth(III) triflate proved to be a powerful catalyst for the dehydration of 7-oxo-15-hydroxy dehydroabietanes. Interestingly, wider studies focusing on the reaction conditions revealed that in apolar solvents, alkenes are obtained, in yields up to 93%, whereas in polar solvents, dimerization occurs and new C-C bonds form, in yields up to 96%. Not only was this method successful for the synthesis of several different 13-propenyl-substituted aromatic abietanes, it also works for compounds from different chemical classes, making this study potentially interesting for a wider research community. With the help of this synthetic method, the semisynthesis of naturally occurring compounds from *Pinus massoniana* was performed here for the first time.

Moreover, we synthesized sets of novel DAA derivatives to be evaluated as potential anticancer agents. Our studies showed that dehydroabietic oximes were able to inhibit the growth of human and murine pancreatic cancer cells with IC_{50} values in the low micromolar range as well as downregulate cyclin D1 expression with upregulation of p27 levels, indicating that the cells are arrested at the G1 phase of the cell cycle. Furthermore, the compounds blocked nitric oxide production and induced differentiation in human leukemia

cells. Screening of the most potent compounds, **173** and **170** against a panel of 140 kinases revealed that **173** is a modest selective inhibitor of RSK2, an AGC kinase, important in the regulation of multiple cellular processes, such as cell proliferation and survival. This study illustrates the potential of dehydroabietic oximes as multifunctional anticancer agents, however, to consider for instance *in vivo* studies, further optimization of the structures is necessary to reach better activities. For instance, replacing the 7-pyridyl group with another heterocycle or position 18 with a longer chain would give a better understanding of the SAR. Moreover, reducing the lipophilicity could be beneficial regarding the bioavailability of the compounds. If targeting the RSK2 would be of interest, testing the binding of a wider set of derivatives and performing some molecular modeling studies could be the next steps worth considering.

To conclude, our studies showed that DAA is a versatile starting material for the semisynthesis of both novel and naturally occurring aromatic abietanes with anticancer activity. All together 41 novel DAA derivatives were synthesized during this work bringing a lot of new information about the possibilities to modify the abietane core and their effects on the bioactivities. Furthermore, we proved that replacement of the hazardous chromium(VI) reagents is possible and hope that, researchers in the diterpenoid field would consider new methods as alternatives for chromium. Overall, our studies illustrate that more research focusing on studying alternative, environmentally sound methods and new catalysts for the semisynthesis of novel diterpenoids is definitely needed.

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